PRINCIPLES AND PRACTICE
OF CHROMATOGRAPHY

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FROM THE PREFACE TO THE FIRST EDITION

"Every scientific advance is an advance in method."

The invention of a new specialised laboratory procedure brings about rapid conquests in new fields of science and technology: finally it exhausts itself and is replaced by a still more practical method. The method of chromatographic adsorption, invented by the talented Russian botanist, Professor M. Tswett, makes possible spatial separation of the components of a mixture. It is just now at the beginning of a brilliant development: it offers a simple experimental procedure to the investigator, especially in the fields of both pure and applied organic chemistry, of biochemistry and of physiology.

It is our hope to assist in the wider application of this procedure, still insufficiently well known. We therefore publish this short monograph, which consists of two parts. First, the fundamental principles of chromatography and the methods of applying them are discussed; there are then given examples of that application of a "Special Part" that is admittedly incomplete. The experimental details brought together here appertain to substances of a very varied nature and may be of use in view of the widely scattered references in the literature. If the field in question is developed further with the desirable speed, then in a measurable time it will be impossible to write an exhaustive account of the subject as it now is to do so for methods of distillation or of precipitation.

The following pages may well often give the impression that the most interesting features are not so much the results that have already been established as the numerous gaps in theoretical and practical knowledge, to help which the readers may be stimulated.

PREFACE TO THE SECOND EDITION

Only one and a half years have elapsed since this book was first published. Yet this new edition embodies notable extensions, shown both in increased scope and additional illustrations, as well as in some 200 new literature references. These in particular show that Tswett's method has made its way into many laboratories where it had previously not been used. Apparently we are now at that stage of development where a new expedient is regarded as a friend in need—although a systematic investigation of its potentialities seldom follows. This is particularly pertinent to the relation between the chromatogram and the constitution of organic components, still insufficiently studied. Schwab and Jockers during the past year have successfully applied the method in inorganic chemistry: the matter is treated in the last chapter.

Comments and information, received during preparation of first and second editions, have been of value: further we are indebted to the following gentlemen: H. A. Boekenoogen (Koog-on-Zaan), H. Brockmann (Goettingen), Ch. Dhéré (Fribourg), H. Fink (Berlin), O. Frehden (Pécs), A. v. Gorka (Pécs), A. E. Gillam (Manchester), I. M. Heilbron (Manchester), G. Hesse (München), P. Karrer (Zurich), R. Kuhn (Heidelberg), E. Lederer (Paris), J. K. Parnas (Lwów), W. Ruhland (Leipzig), G. M. Schwab (München), A. Stoll (Basel), G. Tóth (Pécs), P. Tuzson (Pécs), J. Waldenström (Uppsala), R. Wilstätter (München), A. Winterstein (Basel).

It was our intention to open this book with a biography of Tswett: trustworthy information about the active life of this pioneer has, however, not so far been available to us.

THE AUTHORS.

FOREWORD TO THE ENGLISH EDITION

The translation of this important book into English means something more than making available to British and American chemists information scattered throughout a wide and growing literature : even, however, were that all that the translators and publisher had achieved, there would be innumerable chemists on both sides of the Atlantic who would be grateful to them for their activities.

One of the outstanding merits of this book lies in the fact that Professor Zechmeister and Dr. Cholnoky themselves have made wide and significant contributions to the study of the method with which it is concerned. This deeper acquaintance with the subject has prompted them repeatedly to indicate where further investigation might lead to new practical applications of chromatography, whether in the research or the industrial laboratory. Again, they do not hesitate to speculate, wherever experimental basis affords them justification, on the theoretical implications of the observed facts of chromatography. For these reasons the book makes a distinct contribution to scientific progress and is invaluable not only to those who themselves propose to make use of chromatographic methods, but also to all interested in the sparsely explored territory that lies between the domains of physical and organic chemistry.

Mr. A. L. Bacharach and Mr. F. A. Robinson have not been content merely to produce an accurate translation, but they have also admirably succeeded in retaining in its entirety the stimulating character of the book. I feel confident that, as a result of their efforts, a still greater impetus will be given to the fuller exploitation of this fascinating technique, which has already proved of such inestimable value in various fields of organic and bio-chemistry.

I. M. HEILBRON.

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GENERAL SECTION

CHAPTER 1

PRINCIPLES

"Like the light radiations in the spectrum, so is a mixture of pigments systematically separated on the calcium carbonate column into its constituents, which can then be qualitatively and quantitatively determined."

TSWETT

A survey of progress in organic chemistry and biochemistry shows how often important advances in methods have been made from the physical side. Consider, for example, the developments that have resulted in those branches of science from polarimetry and refractometry, and from spectroscopy in both the visible and invisible regions. It is clear, indeed, that physical methods alone are available for the manipulation of highly labile substances.

To take a specific instance, it is to be noted that the study of organic pigments, and later also of colourless and weakly coloured carbon compounds, was facilitated, to an extent previously undreamt of, by the original proposals for adsorption-analysis, put forward by Tswett in 1906. This simple technique, known as "chromatographic procedure"—more shortly "chromatography"—is the subject of the following pages, which also include a summary of the scientific tendencies set in motion by Tswett's invention.

If a solution of several highly coloured compounds is shaken with a suitable adsorbent, there takes place between the two phases a partition that is determined by mass-relationships and adsorption coefficients. Given favourable conditions, the individual pigments will penetrate more or less deeply into the adsorbent and will be concentrated there. The adsorbate is a mixture and there is no simple way of breaking it up. The conditions are quite different—and much

more favourable—if the solution is allowed to percolate through the adsorbent in one definite direction.

In his fundamental experiments, Tswett extracted green leaves with light petroleum and poured the extract through a compressed column of finely powdered calcium carbonate contained in a vertical glass tube. He found that the apparently homogeneous pigmented contents of the slowly percolating solution underwent separation. In the upper part of the column a pale yellow ring appeared, immediately beneath it two green zones, and farther down three other yellow components, which were fixed only on the more distant portions of the carbonate. Even more pleasing is the behaviour of the " chromatogram " on treatment with adequate quantities of the pure solvent. The picture is then " developed " : white inter-zones appear and increase in width, while the individual pigmented components wander downwards, at different speeds. A yellow compound (carotene) passes through the whole column and is easily obtained in the filtrate.

The composite nature of chlorophyll and leaf-yellow was thus demonstrated and the nature of the components became clearly recognised (Fig. 1, p. 3).

Tswett now cut up the column and dissolved the individual pigments separately in, for example, alcohol. After the decolorised adsorbent had been removed from the separated fractions by filtration, the solutions of the individual pigments were then available for spectroscopic and chemical examination. Thus was a chemist's dream fulfilled : substances that had been mixed together could be cut apart with a knife !

Simple chromatographic procedure consists, then, of the following operations :

Preparation of the column by compression of the adsorbing medium.

Pouring on the solution under investigation.

Development of the finished chromatogram by addition of a solvent.

Expression of the column from the tube.

Cutting up the column according to the layers formed.

Elution of the separate portions of the column.

Separation by filtration of the empty adsorbent, now in powder form.

Further treatment of the individual filtrates (physical measurements, separation of the solutes, elementary analysis, etc.).

Naturally, the *modus operandi* can be modified in many directions—a matter to which we shall return. However much one may vary the scheme, many advantages are as a rule shown over the classical procedure, whereby the solution was treated with the powdered adsorbent. This permitted little insight into the progress of the adsorptions at a particular stage: a glance at the chromatogram, on the other hand, suffices to establish the state of the experiment at any stage.

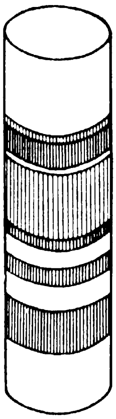


FIG. 1.—Chromatogram of a leaf extract, after Tswett. (In the original the zones are coloured, from above downwards, as follows: pale yellow, green, bright greenish blue, yellow, yellow, yellow.)

The essential advance that we owe to chromatography consists, in short, not in any special selectivity of the adsorbing medium, but in the time and space relationships between solid and liquid phase. Willstätter's famous researches in enzyme chemistry are evidence, indeed, that one can prepare adsorbents of greatly enhanced selectivity and can achieve astonishingly fine degrees of differentiation without the use of chromatography. But only bifurcation of the starting material can be brought about in each individual experiment of this kind, whereas Tswett's procedure can separate numerous fractions from a complicated mixture. Frequently the column shows dozens of layers in the course of a few minutes (Fig. 56, p. 318).

The difference between the individual operations of the two methods can be characterised as follows:

Old Procedure: Adsorption from stationary or stirred solutions. Finely divided adsorbent that is simultaneously permeated in all directions. The adsorbed substances undergo no spatial separation; every particle contains the same adsorbate. Result:

$$\text{Original solution} \begin{cases} \nearrow \text{Total adsorbate} \\ \searrow \text{Filtrate} \end{cases}$$

Tswett Chromatography: Adsorption from a flowing

solution. Adsorbent in columnar form ; defined direction of permeation. The individual compounds, or group of compounds, are spatially separated on the active surface. There are consequently considerable qualitative and quantitative differences between the adsorbate on the individual particles and so also between the different regions of the column. Result :

Original solution
→ Components with highest adsorption-affinity
→ Components with next highest adsorption-affinity
→ Components with still lower adsorption-affinity, etc.
→ Unadsorbed material (in filtrate)

The boundary between the two methods is less sharp if the whole adsorbate is eluted portion by portion : for example, the column can first be treated with a weaker and then successively with stronger and stronger eluents. In favourable circumstances this results in the constituents of the whole " fixed " mixture being dissolved out individually. Even then the use of the Tswett column has the convenience that the eluate flows away on its own.

A transition between the two main methods of carrying out adsorption analysis is also achieved if, instead of constructing a narrow column, one places a layer of the powder on a suction filter and carries out the elution there. One cannot then, of course, rely on following visually what is taking place on the adsorbent. An even closer approach to the older method of procedure is obtained by omitting fractional elutions. This procedure was called " adsorptive filtration " by Fink (1, 2), to distinguish it from ordinary filtration, which depends entirely on a sifting process.

Goppelsroeder's capillary analysis is far inferior to Tswett's method, particularly for preparative work, and especially so if the capillary analytical technique is improved by sucking up pure solvent, as proposed by Schwab and Jockers.

Scope of the Method

It is an essential assumption for the utility of chromatography that the substance bound on the column does not undergo any chemical change during adsorption. This con-

dition is fulfilled in a surprisingly large number of instances, but there are naturally certain limitations to the method. In particular it can happen that a basic or an acidic adsorbent reacts, for example, with formation or decomposition of a salt. Such reversible changes are least disturbing when they are recognised for what they are.

Incidentally we have sometimes observed in working up extracts of organs that the solution in the column takes up mineral matter: for reference to Al_2O_3, see the publications of von Euler and Schlenk.

With sensitive materials, however, it may also happen that the substance undergoes a chemical change in the adsorption column and may be spoiled, especially if operations are carried out too slowly. Some examples of this are given below.

One action of the column is easy to understand, namely the decomposition of addition compounds. Thus it has been observed that certain azulene picrates are broken up on alumina into their components: picric acid remains on the adsorbent, the azulene passes on (Plattner and Pfau, p. 184). A similar cleavage is undergone by an oxidation product of sapogenin (Ochiai, Tsuda and Kitagawa 1, 2; Tsuda and Kitagawa), also by, for example, the picrate of 5-chloro-10-methyl-1 : 2-benzanthracene (Newman 2). Azulene trinitrobenzolate behaves similarly; according to Fieser and Hershberg (5), so does the trinitrobenzolate of 6 : 7-dihydro-20-methylcholanthrene, from which the hydrocarbon $C_{21}H_{16}$ is liberated and passes through with the solvent. Finally, there are similar pertinent observations by Kondo.

Splitting of the acyl groups occurs on the Al_2O_3 chromatogram of diacetyltoxicarol (Cahn and Phipers). According to Ruggli and Jensen, triphenylmethane dyes are bleached on the alumina column, apparently as a result of slow hydrolysis.

Dimerisation has been observed by Hesse (private communication) actually with so simple a compound as acetone. If acetone is sucked through alumina, appreciable quantities of diacetone alcohol are formed $(CH_3)_2COH.CH_2.CO.CH_3$, especially if the oxide has been previously ignited. This is in agreement with the finding of Stoll and Hofmann that part of the solvent from an acetone percolate will distil only with difficulty.

Less clear are the following recorded changes. Chlorophyll can be effectively chromatographed on powdered sucrose, but partial decomposition occurs with, for example, talc (Winterstein and Stein, 2, p. 91). Pterins adsorbed on alumina from weakly acid solutions are rapidly decomposed near the surface of the adsorbent, but they are stable on frankonite (Schöpf and Becker, p. 169). Coloured zones appear on the chromatograms of vitamin A concentrates (Castle, Gillam, Heilbron and Thompson, p. 246). Grassmann has observed that the fluorescence of material adsorbed from tannin extracts changes appreciably in the course of a day or two on the chromatogram (p. 285).

The catalytic action of the alumina column on ethereal oils and the influence of the heat of adsorption have been investigated by Carlsohn and Müller (2) (p. 200).

There is an interesting observation by Kuhn and Ströbele, to the effect that 2-nitro-4 : 5-dimethylaniline after chromatography has lost its ability to form glucosides, as the action of the column has removed from the impure product traces of an essential catalyst, namely ammonium chloride.

For the isomerisation of carotenes, according to Gillam and El Ridi and to Gillam, El Ridi and Kon, see p. 118. For the possibility of *cis-trans* isomerisation, see Taylor and Lavington.

It must be emphasised that, within the limits discussed above, there lies a vast region in which chromatography can be used without difficulty.

Without going further at the moment into details of method, the problems that can be solved by chromatography may be briefly stated, in order to show in what spheres the ordinary technique of the organic chemical laboratory can be supplemented and simplified. This is achieved in the following operations :

(*a*) Testing the homogeneity of a substance.

(*b*) Establishing the identity or non-identity of two substances.

(*c*) Concentration of a product occurring in natural sources at great dilutions.

(*d*) Separation, identification, estimation and isolation of the constituents of a mixture.

(*e*) Purification, e.g. of a technical product, from contaminants.

(*f*) Identification and control of commercial products.

Testing the Homogeneity of a Substance

A substance is homogeneous in the chromatographic sense if it cannot be broken up on the adsorption column. In an extremely large number of instances such homogeneity indicates genuine chemical purity; the strength of adsorption affinity generally depends to a far greater extent on molecular structure than do the indications usually concerned with proof of homogeneity, such as melting-point, boiling-point and spectrum. Differences in solubility of nearly related compounds especially are often slighter than differences in adsorbability. It is therefore desirable whenever possible to replace fractional crystallisation by a chromatographic process or to combine the two procedures.

Because of the high selectivity of surface forces, application of Tswett's principle often makes possible a separation that ordinary laboratory technique fails to bring about.

The following observations may be made as to the limitations of the test for homogeneity. Naturally there are occasions when the compounds possess practically the same adsorption coefficient and cannot be separated on the column. The trustworthiness of the analysis can nevertheless be increased after a negative result if the separation is attempted with other solvents and several adsorbents. For it is unlikely, as Tswett (1) himself remarks, that the adsorption isotherms of two substances would change in a strictly similar way when the medium and the adsorbing phase are varied at will.

Proof of homogeneity can, moreover, be taken a little further. The undifferentiated layers can be cut into several zones, which can be individually further tested by physical or chemical means. It occasionally happens that differential adsorbability permits separation of substances, but is not sufficient for the formation of an empty inter-zone.

In rare instances another difficulty may be met: a simple substance may give a heterogeneous picture on the column. This can be brought about, as stated above, by the formation

or decomposition of a salt, which has sometimes been observed : it happens, for example, according to Karrer and Strong (p. 178), with anthocyanins. Further it is to be expected on physical grounds that strongly polydisperse solutions will not show chromatographic uniformity. More definite investigation of these conditions is needed. (Apparent homogeneity, p. 74.)

Establishment of Identity or Non-identity of Two Substances

When this question has to be decided, a solution is prepared of the two substances together and this is submitted to adsorption analysis. The formation of two zones indicates non-identity ; identity leads to no differentiation on the column. The mixed chromatogram should replace determination of the mixed melting-point ; in favourable circumstances it requires scarcely more material than the old-established test for identity.

If a solution yields two layers on adsorption and if it has to be decided which of the two is identical with a known substance, a larger amount of the latter is added to the solution. It can then be established whether the lower or upper zone in the chromatograph has been appreciably widened in comparison with the appearance of the original column.

Concentration of a Product occurring at Great Dilution in a Natural Source

In biochemical investigations there is no process as effective for this purpose as chromatography. Several litres or several hundred litres of solution are put through a relatively small column of a suitable substance and the filtrate is rejected. In this way the scale of operations is diminished at one stroke without the need for evaporation, which is both time-consuming and undesirable for labile substances. Generally the whole of the retained material is eluted without further separation, to allow of more precise examination ; alternatively the components can be dissolved out separately. The concentrate can be worked up further by chromatographic or other methods.

Example : Isolation of urinary pigments (Koschara, 1 to 5 ; p. 175).

PRINCIPLES

Separation of a Mixture : Identification, Estimation and Isolation of the Components

This is the main purpose of chromatography : it allows of the solution of manifold problems, and involves many kinds of technique. These can only be sketched here in brief.

As is well known, analytical organic chemistry is increasingly concerned with small quantities of material. The powerful impetus given by the introduction of Pregl's micro-analysis, particularly to biochemistry, is having most fruitful results ; by its means the determination of molecular weight, as well as the identification of elements and atomic groups, can be carried out with only a few milligrams of material. The modern weapon of micro-analysis could, nevertheless, not be fully used as long as knowledge was not generally available of a method for preparing in pure form small quantities of a substance, by its separation either from similar compounds or from a complex mixture. Fractional crystallisation of very small quantities will generally not lead to the desired goal, and it often offers no means for testing the homogeneity of the end-product.

This methodological gap has been bridged in the happiest manner by the Tswett procedure. Substances prepared by chromatography and proved homogeneous on the column are ready for quantitative micro-analysis.

Naturally the scale of operations can be increased ; work is already being carried out in some laboratories with batches involving the use of several kilograms of adsorbent. There is no obstacle, under favourable conditions, to the application of Tswett's procedure in manufacturing processes, and an extension of the patent literature in that direction can be forecast with certainty.

The more complex the mixture, the more difficult is a clean-cut separation. This can, however, be improved and the desired end achieved by further chromatography, repeated with the individual fractions.

In laboratory practice it is sometimes impossible to isolate small amounts of solute contained in homogeneous fractions, as when, for example, there is not naturally present even enough material for micro-analysis. One may in this and other instances content oneself with identification and estimation

of the material in the eluate. All customary procedures are available for this purpose. The advantages of the methods discussed here consist just in this : that one can work almost without losses, at least in the simpler cases, and can eventually undertake any appropriate chemical or physical operations.

For dyestuffs, spectroscopy and colorimetry are available ; with pale or colourless substances other, in some circumstances biological, methods are needed.

Purification, e.g., of a Technical Product, from Contaminants

It not seldom happens that " purest " commercial chemicals are very far from homogeneous in a chromatographic sense. The more stages that were necessary in the manufacture, the greater the probability of heterogeneity in the end-product.

For purification it often suffices to undertake a simplified procedure, whereby only the main zone is worked up, the other portions of the column being rejected. If it is intended to carry out complete purification, then the adsorptions must be repeated until there is no further formation of subsidiary zones. Even when the column shows empty zones so narrow that sharp separation cannot be accomplished by cutting the column, the degree of purity can be increased by repeating the chromatography, as the absolute amount of contaminant falls rapidly.

The experiment is especially simple and impressive when a pigment is to be separated from a preparation of which the main constituent is colourless, and the fate of the pigment can be followed by eye, or when the adsorption coefficients of main and subsidiary products are so widely different that one of the two runs easily through the column and arrives in the filtrate quantitively.

Here also technical vistas are opened for manufacturing processes of purification by adsorption, in close association with procedures already in use.

Examples : p. 209.

Identification and Control of Commercial Products

A commercial product of particular origin and quality, in so far as it can be dissolved or extracted, has a characteristic chromatogram, so that there are manifold expedients available

for commercial analysis, and also for practical pharmacy. The task here is not to effect a separation, but to obtain the clearest possible empirical identification of the material and the exposure of falsification. In the course of such adsorption analyses, one frequently uses ultra-violet illumination of the column, as described on page 81 *et seq.*

One of the first applications of this kind is found in the work of Grassmann and of Grassmann and Lang (p. 285). The chromatograms prepared by them were intended in certain instances for the rapid differentiation of tanning materials from various sources. Attention is also drawn to the applications by Ruggli and Jensen (1, 2) in connection with the evaluation of certain coal-tar dyes. Chromatographic indications of wine sophistication, page 181 (Mohler and Hämmerle): investigation of technical oils and fats, page 287 (Boekenoogen; Thaler); of galenicals, page 291 (Valentin and Franck; Merz and Franck).

Historical

In 1910 there appeared Tswett's (1) comprehensive work "Chromophylls in Plant and Animal World"; at that time chromatography, introduced in 1906, was hardly used at all outside the laboratory. Tswett quotes only the work of Kränzlein, and of Stoklasa, Brdlik and Ernest, which were partly polemical. As already pointed out, the whole development of the new method derived from the study of the green pigments of leaves. So convinced was Tswett of the scope of his discovery and of the trustworthiness of the procedure that he insisted—although his experiments did not result in the isolation of pure substances—against all opposition that chlorophyll was a mixture of two components, as also the "phaeophytin" of that period. On the basis of chromatographic observations Tswett also declared that the so-called "crystalline chlorophyll" did not occur naturally and must consist of two components. He recognised that the difference between crystalline and amorphous chlorophyll had nothing to do with the duality of the natural pigment. (Tswett, 1, 4, 8, 10, 13, 15, 16, 20, 21.)

In the following decades, the new experimental technique remained practically unnoticed, partly because of the unfor-

tunate circumstance that Tswett's above-mentioned book had only been published in Russian. Although Willstätter (3) already in 1910, then Willstätter and Stoll, and also Willstätter and Isler (1912) referred emphatically to the significance of Tswett's work, one can regard the quarter of a century from 1906 to 1931 as the latent period in the history of chromatography.

A few isolated discussions, referred to below, date from this early period. One of the first investigators to use Tswett's method was Dhéré, in whose laboratories methods of adsorption analysis had been practised since 1911. He made his pupils Rogowski and Vegezzi carry out experiments on, among other substances, the constituents of the green-leaf colouring matter and the old " Tetronerythrin." At the same time important biochemical researches were reported by Palmer, mostly with Eckles, in connection with chromatographic investigations of the pigments of milk-fat, as well as of other vegetable and animal products. Palmer (1) also, in his monograph in English, called special attention to Tswett's ideas (1922).

Rather later investigations were made by Coward (1924) and then by Lipmaa (1926), concerned respectively with flower extracts and the separation of rhodoxanthin and xanthophyll.

The efflorescence of chromatography dates from 1931 and is characterised above all by modernisation of the technique and by transposal of the experiments to the preparative scale.

Tswett worked with small amounts of material and made no attempt to precipitate the dissolved pigments. Their crystalline nature was hardly known, nor did they ever allow of chemical analysis. Consequently some useful observations remained unnoticed or were tacitly doubted. That Tswett (1) himself, however, was aware of the preparative possibilities of chromatography is shown, among other things, by his experiments in which the separation of " lecithin " crystals from fresh egg-yolk was accomplished.

Such isolated observations remained, however, without more general effect on chemical practice. Tswett's method only achieved actuality at the moment when the classical enzyme researches of Willstätter and his school made it clear that surprisingly fine differences of structure can be demonstrated by systematically conducted adsorptions and elutions.

At the beginning of 1931 Kuhn and Lederer, and also Kuhn, Winterstein and Lederer, successfully introduced chromatography into the preparative chemistry of the polyene pigments. The first work was concerned with the splitting of carotene, then known for just one hundred years, into its components, as well as with the isolation of α- and β-carotene : the latter authors were also able to obtain lutein from various vegetable tissues and to separate the components of egg-yolk pigment. Thus in 1910 Tswett's prophecy was fulfilled : "Very likely leaf carotene is not a chemical entity, but a mixture of two or more homologues, which it may be possible to separate from one another by means of the adsorption method, with the use of suitable adsorbents."

Further, at the end of 1931, Petter, independently of the above-mentioned investigators, undertook the sub-division of bacterio-ruberin, also on a preparative scale.

In recent years, in the hands of Karrer, as well as of Kuhn, Winterstein and others, chromatography for preparative purposes has given a new stimulus above all to the chemistry of the carotenoids, and it is impossible to conceive its banishment from this chosen field. The experiments have been conducted not only with higher plants, but also with the pigments of algae, fungi and bacteria (Chargaff, Heilbron, Karrer, Lederer, Willstaedt, and others), as well as with animal lipochromes (Brockmann, v. Euler, Heilbron, Karrer, Kuhn, Willstaedt, Winterstein, Zechmeister and Tuzson, and others).

It was a short step also to submit the most important plant pigment, leaf-green, to modern treatment. Chlorophylls *a* and *b*, already recognised by Tswett, were first isolated by Willstätter and Stoll and most closely investigated by them : they were prepared in a high degree of purity by Winterstein and Stein (2, 3) by chromatographic means. Mention must be made of the researches into bacterio-chlorophyll and its degradation products, by H. Fischer and others. As will be shown later, decisive advances were made in the sphere of the physiologically important flavins (Karrer, Koschara, Kuhn) as well as of other natural pigments. The fractionation of the water-soluble anthocyanins has recently been achieved by Karrer and Strong.

Qualitative experiments with artificial dyestuffs had

already been made by Tswett; he separated, for example, commercial Oil Blue and Sudan III into several zones from a solution in light petroleum. Ruggli and Jensen (1, 2) worked with water-soluble and other aniline dyes.

An inexhaustible field, but one only recently opened up, is the investigation and fractionation of colourless substances on the column. In this connection are to be found exploratory experiments of Tswett (1), but they were not very successful and have been forgotten. Recently various devices have been introduced into the technique, in order to make the zones apparent, so that the progress of the experiment can be better followed and the planned cutting of the column carried out. Of a similar nature are colour-reactions with the filtrate or eluate, or those carried out on the column itself. Especially worthy of development is illumination of the adsorption tube with the mercury lamp, which often makes it possible to distinguish differently fluorescent substances (" ultra-chromatography "). This method has been recommended both by Winterstein and Schön (1) and by Karrer and Schöpp (2) in 1933 and 1934.

Water-soluble substances can be handled by chromatography, but this generally involves some suitable modification in procedure. Koschara is to be thanked for statements of guiding principles on this subject (see also Fink).

It has been our intention in the foregoing matter to give only the broad outlines of historical development. In the Special Section will be found more detailed accounts of the method's manifold applications. From these it will be seen that adsorption procedures have provided an indispensable aid to innumerable syntheses. In connection with substances of biochemical and biological importance, the greatest advances will have to be recorded in the vitamin field. As is well known, Karrer (with Morf and Schöpp) obtained pure vitamin A preparations by chromatography (see also the researches of Heilbron and of Holmes and their associates). Vitamin D_3 has lately been prepared artificially by Windaus, Schenck and Werder and by Brockmann (3) from fish-liver oils, using chromatographic methods. Later applications to vitamin and hormone chemistry will be found in the Special Section (pp. 243–279). In the sphere of enzymes, co-enzymes, bio-

chemical activators and so on, wide opportunities present themselves to the investigator (cf. Euler and Adler ; Euler and Schlenk, 1, 2).

Finally it may be observed that in 1937 Schwab and Jockers (1, 2) successfully introduced chromatographic concepts into inorganic chemistry (see also pp. 304–316).

Processes must also be occurring in nature that, leading to local increases in concentration of substances, are comparable with the unidirectional passage of liquid through the Tswett column (cf. model experiments by Lou). For Tswett's fundamental idea is not the straight form of the column, but the fixed, even though non-linear, direction of the flow. If this condition is fulfilled, there becomes possible the local selection from the fluid of substances in accordance with their adsorption affinities.

Furthermore, according to Treibs, certain geological processes can also be considered from this point of view (see also Schwab and Jockers).

The widely scattered literature has markedly increased in recent years, as can well be seen from the list of references in this book. The first modern extensive summary is due to Winterstein (1), and Willstaedt (12) announces a monograph. Shorter bibliographies have been furnished by Armstrong, Celsi, Coffari, Cook, Dam, Hesse, Koschara (6), Lederer (1), Schwab, Sörensen (3), Stix, Valentin, Willstaedt (1) and the authors of this book (*v.* also Zechmeister, 1, 6). It is a matter of satisfaction that the chromatographic method has already gained entry into a number of practical text-books designed for students' use.

THEORETICAL

The theoretical foundations of chromatography were already recognised by its discoverer. It is apparently a matter of selection, based on the adsorption affinities of several substances, in a common solution, showing different degrees of activity to the same adsorbent. The individual components form layers in a descending order, corresponding with the diminution in their surface activities. The various individual stages in chromatography as practised, often adopting Tswett's (1) method of approach, will be surveyed in what follows. For simplicity we shall follow the fate of a

mixture of pigments, but we must emphasise that chromatography of colourless substances obeys the same fundamental course.

If the capacity of a given amount of adsorbent is regarded as constant, all the substances in solution will compete simultaneously in these surface activities, but with very different end results. Should the amount of the adsorbent be insufficient to hold all of the pigments, then only the colouring matter with the strongest adsorption affinity will be retained, thereby "exhausting" the adsorbing surface.

This is indeed what takes place when the solution is poured on to the column and comes in contact with the first layer, to be envisaged as sufficiently thin. By passing through this the solution is therefore only diluted in respect of its best adsorbed constituent. Otherwise it is unaltered, and passes now into the next region, where the rest of the substance in question is gradually exhausted and retained. Its part is then played by another pigment, which has a lower adsorbability; its concentration steadily diminishes as the liquid continues to flow, so that this second pigment also is deposited in the column. So the play continues, until the solution becomes practically colourless; finally there is to be found in the sequence of coloured zones formed a scheme of adsorption-sequence comprising all the components of the original solution.

The above description of the consecutive stages in the formation of a chromatogram is only satisfactory in its broad outlines, and needs expansion to include detailed consideration of the more intimate processes.

Apparently it is not strictly correct to assume that the surface of every particle immediately and exclusively adsorbs those components endowed with the highest affinity. One can envisage that in the uppermost region of the column the solid phase exhibiting surface activity comes suddenly into contact with the complex solution; thus at the first moment every particle is surrounded with molecules of all the pigments. All these molecules make immediate contact with the unoccupied surfaces of the particles and take part in the formation of an adsorbate. Thus pigments with relatively lower adsorption affinities at first adhere to the unit layer, but they are *displaced* from their adsorption complex by the "stronger"

components that immediately follow; they leave the solid phase and return into solution. After their liberation they are carried by the stream of liquid to the next lower-lying region, where they again undergo displacement after a transitory adherence. Every constituent deposits itself permanently only if nothing "stronger" follows, that is, when the coloured zones of all substances that can be reckoned as displacers are already completed in higher positions on the column.

Experimental evidence for these considerations dates back to Tswett. We ourselves have also had frequent opportunities of observing these characteristic relationships. For example, a solution of a homogeneous yellow colouring matter was introduced into the column and remained fixed near the upper surface. When a stable chromatogram had been formed, we poured in a second solution, containing a red compound with a higher adsorbability. As a result the first coloured ring forthwith wandered down the column, its place being taken by the second pigment. (Figs. 52, 53, p. 317.) If the experiment is repeated in the reverse chronological order, a displacement of this kind does not take place: the solution of the less adsorbable yellow pigment, introduced after the other, now passes through the region in which the red is already fixed and occupies a lower position. In both instances the same chromatogram results. Ruggli and Jensen (1, p. 37) have had similar experiences with azo-dyes.

Under any given experimental conditions, therefore, only the serial order of the individual substances is fixed, whereas the absolute position of each pigment on the adsorbent is determined by the nature of the others.

Interesting also are the stages occurring in the "development" of a chromatogram. When pure solvent is sucked through, pigment is displaced from the active surface, that is to say, dissolved, according to the individual adsorption coefficients. Partition between the two phases contributes to the adsorption equilibrium in question. When this is reached, the solution leaves the individual zones unaltered as to the concentration of each particular pigment. But the moment it encounters a region free of pigment, adsorption begins anew and the dissolved pigments are eventually completely trapped

by what can be regarded as a continuous series of thin layers. Then the colourless developing solvent flows on, its pigment content having gradually increased from zero to a limiting value and then fallen again to zero, in the course of the stages described.

By this process of development the picture on the column can be altered to various extents and at quite different speeds, according to the magnitude of the operating forces of adsorption. If there is only weak adsorption, then the adsorbed substance remains only transitorily on the column and is very rapidly washed into the filtrate by the selected solvent. The opposite limiting case occurs when the adsorption is exceedingly strong and therefore practically irreversible, so that washing, for however long, with the solvent used during adsorption is without effect. In such circumstances the relation between adsorbent and adsorbed substance approximates to chemical combination, in the classical sense, although no sharp line can be drawn between these two concepts since the establishment of the electronic theory.

The stronger and the more irreversible the attachment of a substance to the column, the more must development depend upon the use of a second solvent. In the instances investigated by Carlsohn and Müller (1, 2) the adsorbed material could only be liberated by such solvents as were themselves more strongly adsorbed. Under these conditions, therefore, there is also a displacement, which characterises the method of Tswett.

The stages analysed in detail above are shown simultaneously at innumerable points in the adsorption column. Every unit volume of the washing liquid dissolves out fractions of the pigment and transports them to a lower region of the column. These stages are integrated into a slow descent of all the pigments. It is clear that the progress of the individual zones, determined by the differences in adsorption coefficient, will begin and proceed with unequal velocities. Generally the most weakly adsorbed and most easily eluted components will be carried down most rapidly. As, however, just these pigments have originally formed the lowest layers of the crude chromatogram, the colourless inter-zones will either increase during development or will actually only manifest

themselves then for the first time ; the individual layers will become more sharply demarcated and defined, much to the advantage of the experimentalist, who has then merely to cut up the column after its removal from the tube.

The following observations may be made about the subsequent elution of the individual pigments from the different portions of the column. A suitable medium for the purpose must be selected empirically. Carbon disulphide, benzene, light petroleum, carbon tetrachloride may have been used in the initial stages of the experiment, in which event, according to Tswett, the addition of ethanol will often effect immediate elution. It is, of course, not always to be expected that a substance will be easily adsorbed from a medium in which it is sparingly soluble, or that good solvents will be effective for elution. Often precisely the contrary condition prevails ; carotene, for example, in petroleum solution is strongly held by the chromatogram and eluted by addition of a little alcohol, although it is relatively soluble in the former solvent and much less soluble in the latter. It is here a matter not of simple solubility phenomena, but of the breaking up of an adsorbate, in which both physical and chemical influences collaborate.

Prospects

Although the phenomena manifested in the column are understandable, at any rate as to their nature, a deeper insight into the problems of the concepts involved is to be desired, in relation to the recognised physico-chemical theory of adsorption.

Connections between the behaviour of a substance in the Tswett column and its physical constants could be established, as, for example, the viscosity of the solution, which diminishes steadily during the course of the experiment. There must also exist a relationship with the diffusion velocity and, further, with fastness to fibres. The last problem was attacked by Ruggli and Jensen (1). In many instances examined, but not in all, it was found that coal-tar dyestuffs were the more easily bound by alumina the more slowly they diffused in gelatine. But a rule of general validity is not yet established.

There is, in fact, a lack of theoretical foundations, adequately developed ; even for the special case of the Tswett procedure this can only be achieved within the frame-work of the electronic theory. The conditions for stable adhesion in the column are dependent on the fine molecular structure of the solvent, among other things ; for example, vitamin A is taken much more easily from the homopolar cyclohexane than from the heteropolar chloroform (Bowden and Bastow).

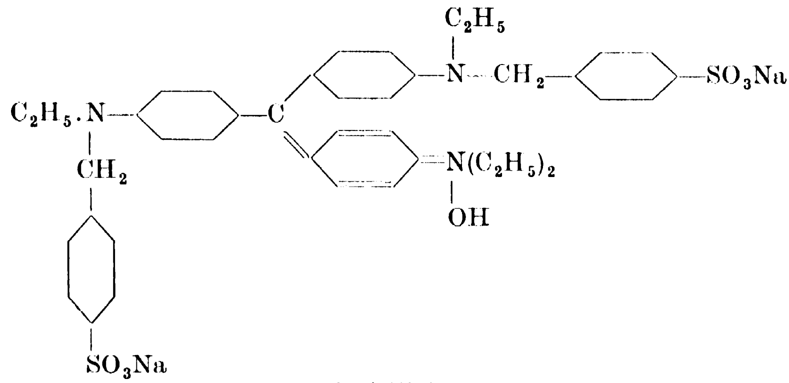
A further task consists in removing from the sphere of pure empiricism the behaviour of adsorbents prepared in different ways (cf., for example, the study by Carlsohn and Müller, 1, 2 ; p. 200).

Particularly promising problems are those concerned with questions of elution, especially the processes of displacement, even though the method of attack is by no means easy to foresee. In the simplest instance it is a question of interaction between adsorbent, solvent and two dissolved organic compounds. Not only is the velocity of displacement dependent on the constitution of the solid phase, but also its direction ; thus—to take one example from many—oestrin takes its place above indirubin on a column of calcium hydroxide, whereas on alumina the order of the zones is reversed (Duschinsky and Lederer). The result is affected by the existence of more or less permanent chemical relations between adsorbent and adsorbed substance, for example, the formation of salt-like associations, complex " compounds " or resins. The theoretical study of more involved processes—the " multi-component " problem in chromatography—would also seem opportune.

Exchange Adsorption

A special kind of displacement takes place when, simultaneously with the adsorption, certain groups of atoms are removed from the solid phase to make way for the substance to be taken up. A pretty example of this is lead sulphate, studied by Kolthoff, v. Fischer and Rosenblum (*v.* also Kolthoff and Rosenblum). If this adsorbent binds the triphenylmethane dyestuff, Wool Violet 4BN, sulphate ions are *exchanged* for dyestuff ions on the surface of the solid phase :

$$\underset{\text{(surface)}}{PbSO_4} + \underset{\text{(solution)}}{\text{Wool Violet}} \longrightarrow \underset{\text{(surface)}}{\text{Pb-Wool Violet}} + \underset{\text{(solution)}}{SO_4^{--}}$$



Wool Violet 4BN

The former pass into the solution, where sodium ions from the dyestuff also remain. Evaluation of such observations in the chromatography of other organic substances is desirable. Interesting accounts of the behaviour of inorganic ions are given by Schwab and Jockers (1, 2) and also by Schwab and Dattler.

If a suitably chosen metallic salt, for example, copper sulphate, is poured on to an aluminium oxide column, then the copper is easily fixed, but not the sulphate ion, which appears in the effluent. Consequently an amount of a cation equivalent to the sulphate ion must be displaced from the material of the column into the liquid phase. The filtrate does, in fact, contain a metallic ion ; not, however, aluminium, but sodium, which appears to be present even in the purest kinds of adsorbent. During the experiment other metals displace it by a " permutit " kind of action ; for example :

$$\underset{\text{(surface)}}{\text{Sodium aluminate}} + \underset{\text{(solution)}}{Cu^{++}} \longrightarrow \underset{\text{(surface)}}{\text{cupric aluminate}} + \underset{\text{(solution)}}{2Na^{+}}$$

Indeed, cobalt or copper ions are less strongly adsorbed by columns of sodium-free aluminium oxide than by the commercial grades. When the chromatogram is developed, moreover, the zones are observed to be more widely spread (Fig. 2). In accord with this is the observation of the authors cited, namely, that unoccupied inter-zones do not occur in such instances ; once the sodium has been displaced, the adsorbent never becomes " empty " again.

If the column has received a preliminary treatment with dilute nitric acid, the original content of alkali is removed. The portions adapted for adsorption must now contain aluminium nitrate, of which the anion can be displaced by "stronger" anions. Such a column is suitable for the exchange-adsorption of certain acid radicals (p. 314).

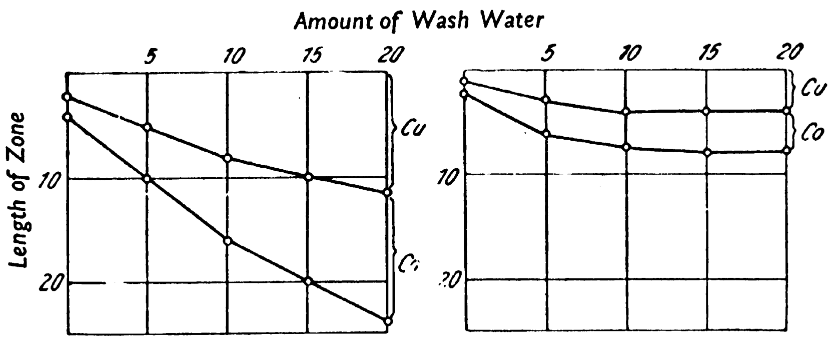


FIG. 2.—Growth of zones on washing chromatogram with water (Schwab and Jockers, 2). Left, alumina poor in sodium: right, ordinary alumina.

To summarise, physico-chemical investigations of the particulate phenomena especially characteristic of the adsorption column still need to be elaborated.

THE RELATIONSHIP BETWEEN CHROMATOGRAM AND CONSTITUTION

It is unsatisfactory merely to establish empirically the behaviour of innumerable compounds on the Tswett column; the connection with molecular structure must be more closely investigated. Although not much progress has been made in this subject, there are already available certain bases for experiment—namely the predictions in certain instances of adsorption behaviour. Sometimes also deductions as to structural formula follow from chromatographic investigation.

Chromatographic Separation of Stereoisomers

It is important to assess the value of the Tswett procedure in stereochemistry. On the one hand it makes it possible to demonstrate the extent of structural differences, necessary for

the separation of two substances, and on the other it affords the possibility of isolating substances that are to be obtained in no other way.

Winterstein and Stein (1) and Winterstein (1), in the first place, have briefly reported the chromatographic fractionation of *cis*- and *trans*-bixin and of *cis*- and *trans*-crocetin dimethylester, on the adsorption column. Among nitrogenous substances the *cis* form of azobenzene, produced photochemically, according to Hartley (1, 2) can be separated on the chromatogram (Cook ; Zechmeister, Frehden and Fischer Jörgensen ; Al_2O_3 ; benzene. Fig. 68, p. 322). Recently Cook (2) has published a detailed treatment of the same subject. In the sphere of optical isomerism Stoll and Hofmann have described the separation of epimers (*v.* also p. 241). An especially interesting result is the partial resolution of a racemate carried out by means of a column filled with an optically active substance (Henderson and Rule). The highly selective nature of adsorption affinity has thereby received a further beautiful illustration. These authors poured a weak solution of *p*-phenylene-bis-imino camphor, $C_{10}H_{16}{=}N.C_6H_4.N{=}C_{10}H_{16}$, in petroleum-benzene mixture (8 : 1) through a column of lactose. Development was carried on with the same mixed solvent until the pale yellow zone, at first fixed near the top of the column, filled practically the whole tube. The column was then cut into four, and the chloroform eluates were examined polarimetrically. It was found that the dextro form had been more strongly adsorbed than the lævo, for the material held in the upper quarter of the tube had a specific rotation (in chloroform) for the sodium line of $+$ 90°, whereas that from the lowest quarter gave $-$ 50°. These values could not be altered by recrystallisation. As the value of $[\alpha]_D$ is $\pm$ 1500° for the antipodes, the resolution of the racemates had here been achieved to the extent of one part in sixteen of the dextro component.

Chromatographic Separation of Related Compounds

There now follows information as to the influence of certain atomic groupings on the strength of adsorption affinities, in so far as this is available at the present time.

1. Synthetic Polyenes

The polyenes contain a longer or shorter system of successive conjugated carbon double bonds in an open chain. The representatives of this series are built up in a strictly analogous manner, so that it is easy to ascertain which part of the structure is specifically responsible for increases in adsorbability. It is the ethylenic bond. The longer the unsaturated grouping mentioned, the stronger does the adsorption affinity prove, which is theoretically what must obviously occur. Thus the diphenyl polyenes prepared by Kuhn and Winterstein (5), having the general formula, $C_6H_5—(CH=CH)_n—C_6H_5$, show, according to Winterstein and Schön (2), the conspicuous influence exerted by the addition of a single double bond on behaviour on the adsorption column (Table 1).

TABLE 1

ORDER OF ADSORPTION OF THE DIPHENYL POLYENES

Most strongly adsorbed	$C_6H_5—CH=CH—CH=CH—CH=CH—CH=CH—C_6H_5$ Diphenyl-octatetraene
↓	$C_6H_5—CH=CH—CH=CH—CH=CH—C_6H_5$ Diphenyl-hexatriene
↓	$C_6H_5—CH=CH—CH=CH—C_6H_5$ Diphenyl-butadiene
↓	$C_6H_5—CH=CH—C_6H_5$ Stilbene
Most weakly adsorbed	$C_6H_5—C_6H_5$ Diphenyl

After development the homologues mentioned take up clearly differentiated positions on the chromatogram, and this can be most prettily brought out in ultra-violet light ; above are to be seen two different yellow zones, below three zones with different shades of blue fluorescence.

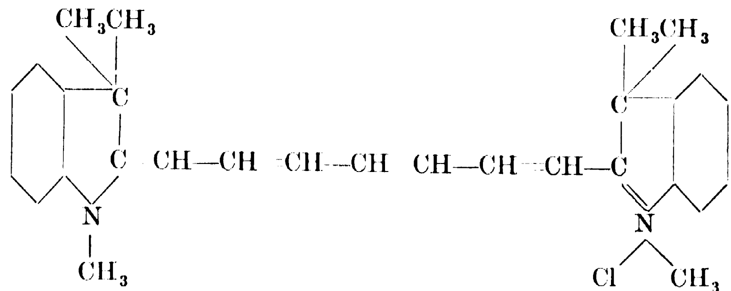
When the benzene rings are replaced by certain heterocyclic structures the influence of the conjugated double-bonds on increased adsorbability still remains. According to Ruggli and Jensen (1) the alumina chromatogram prepared from an aqueous solution of compounds in the indolenine series, studied by Kuhn, Winterstein and Balser (results partly unpublished),

holds the blue dyes in the top and the yellow in the bottom portions. Here also depth of colour and adsorption-affinity run parallel (Table 2).

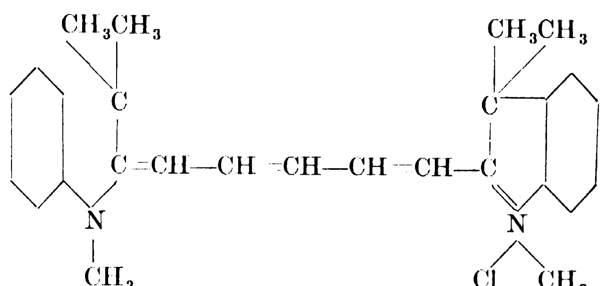
TABLE 2

Order of Adsorption of Certain Indolenine Dyes

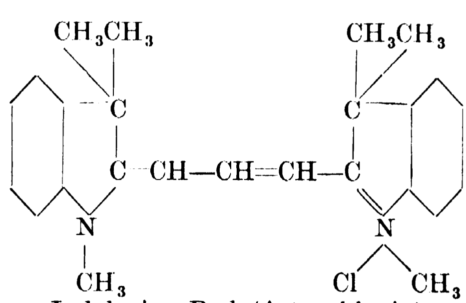
Most strongly adsorbed



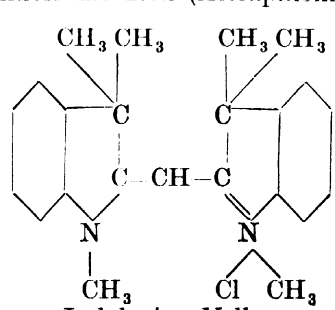
Indolenine Blue



Indolenine Violet



Indolenine Red (Astraphloxin)



Indolenine Yellow

Most weakly adsorbed

2. Natural Polyenes : Carotenoids

As is well known, the carotenoids are yellow to violet pigments, soluble in fats and insoluble in water : they are derivatives of dehydrogenated isoprene residues. The whole or partially aliphatic nature is expressed in the symbols (0, 1 or 2 ionone rings) used on pages 29 to 31. The carotenoids are distinguished from the synthetic polyenes, discussed above, by the branch nature of the carbon skeleton. (For further details, see the monographs of Zechmeister 1, 6 ; Willstaedt, 2 ; Lederer, 2, 3, 12.)

The carotenoids, in the narrower sense of the term, those with 40 carbon atoms, are suitable material for investigations into the relation between structure and behaviour on adsorption. The general configuration of the molecule is identical in the various representatives of this class of pigment, as are also the position and linkage of the 22 middle carbon atoms. From the structural formulæ (Tables 5 and 6, pp. 29–31) the following rules may be established. According to our knowledge to date, the adsorption affinity increases, that is, the place taken in the column is higher, with compounds otherwise analogous when

(1) the number pf double-bonds increases (e.g., lycopene and γ-carotene) ;

(2) of a given number of double-bonds, all are conjugated (e.g., α- and β-carotene) ;

(3) hydroxyl groups occur in unsaturated systems otherwise identical (e.g., kryptoxanthin and β-carotene) ;

(4) the number pf hydroxyl groups increases (e.g., zeaxanthin and kryptoxanthin)

and (5) a carbonyl group is conjugated with a carbon double-bond (e.g., capsanthin and zeaxanthin).

These rules are true not only for the natural carotenoids, but also for their artificial degradation products, such as carotenone and semi-β-carotenone (Kuhn and Brockmann, 6, 10 : Table 7, p. 32). For the relation between colour and behaviour on adsorption, see pp. 35, 117.

The tenacity with which a polyene is held on the adsorbent surface is, therefore, found to go parallel with those forces that were grouped together in the older chemistry under the

concept "partial affinity." Here is also to be recognised the specific influence of the hydroxyl group, whose effect, for example, with $CaCO_3$, $Ca(OH)_2$ or Al_2O_3 as adsorbent is actually much more marked, in a chemical sense, than that due to the increase of one double bond. Thus lutein with only 10 conjugated F takes a higher place in the column than lycoxanthin with 11 conjugated F, to which result the larger number of hydroxyl groups, two against one, contributes decisively (Table 6, p. 31). The remarkable position of rhodoxanthin is also to be seen there. This ketone with 12 conjugated F and two adjacent carbonyl groups, but without hydroxyl groups, is lower down than a polyene alcohol with only 11 carbon double-bonds. Here also one recognises the particularly marked specificity of the hydroxyl group for the adsorbent.

When the hydroxyl groups are esterified, the adsorption affinity of the carotenoids shows an extraordinary fall, so that the behaviour becomes reminiscent of that of carotenoids containing no oxygen. In the series not only does zeaxanthin dipalmitate (physalien) fall below zeaxanthin but its adsorbability is actually exceeded by that of kryptoxanthin; an unesterified hydroxyl group is thus far more effective than several esterified ones. Furthermore, the greater length of the conjugated system is at once manifested in rhodoxanthin again, if it is compared with, for example, free and esterified zeaxanthin: in the column the ketone occupies an intermediateposition.

The chromatographic behaviour of esters, in particular, is also conditioned by the nature of the acidic component, especially if this is unsaturated.

The first order of adsorption in the sphere of the carotenoids was given by Winterstein (1) and is reproduced in Table 3, p. 28.

If one turns also to some of the more recently discovered representatives, the following approximate sequence of zones is found for the unesterified C_{40} carotenoids containing oxygen (Table 4): among these certain pairs of compounds have been arranged in an order deduced from the literature, as they could not be directly compared in the column—fucoxanthin—capsorubin, lycophyll—flavoxanthin, antheraxanthin (and petaloxanthin)—flavoxanthin, the same—lycophyll.

TABLE 3

ORDER OF ADSORPTION OF THE MORE IMPORTANT CAROTENOIDS FROM PETROLEUM SOLUTION. (WINTERSTEIN, 1)

Most strongly adsorbed	Fucoxanthin	$C_{40}H_{56}O_6$	Alcohols	$CaCO_3$
	Violaxanthin	$C_{40}H_{56}O_4$		
	Taraxanthin	$C_{40}H_{56}O_4$		
	Flavoxanthin	$C_{40}H_{56}O_3$		
	Zeaxanthin	$C_{40}H_{56}O_2$		
	Lutein	$C_{40}H_{56}O_2$		
	Rhodoxanthin	$C_{40}H_{50}O_2$	Ketone	Al_2O_3
	Physalien	$C_{72}H_{116}O_4$	Esters	
	Helenien	$C_{72}H_{116}O_4$		
	Lycopene	$C_{40}H_{56}$	Hydro-carbons	
	γ-Carotene	$C_{40}H_{56}$		
Most weakly adsorbed	β-Carotene	$C_{40}H_{56}$		
	α-Carotene	$C_{40}H_{56}$		

TABLE 4

ORDER OF ADSORPTION OF UNESTERIFIED CAROTENOIDS CONTAINING OXYGEN

Most strongly adsorbed	Fucoxanthin	$C_{40}H_{56}O_6$	(Structure unknown)
	Capsorubin	$C_{40}H_{60}O_4$	(Dihydroxy-diketone)
	Capsanthin	$C_{40}H_{58}O_3$	(Dihydroxy-monoketone)
	Violaxanthin	$C_{40}H_{56}O_4$	(Tri- or tetrahydric alcohol)
	Taraxanthin	$C_{40}H_{56}O_4$	
	Antheraxanthin	$C_{40}H_{56}O_3$	(Tri-hydric alcohol)
	Petaloxanthin	$C_{40}H_{56}O_3$	
	Flavoxanthin	$C_{40}H_{56}O_3$	
	Lycophyll	$C_{40}H_{56}O_2$	(Di-hydric alcohol)
	Zeaxanthin	$C_{40}H_{56}O_2$	
	Lutein	$C_{40}H_{56}O_2$	
	Lycoxanthin	$C_{40}H_{56}O$	(Monohydric alcohol)
Most weakly adsorbed	Kryptoxanthin and Rubixanthin	$C_{40}H_{56}O$	
	Rhodoxanthin	$C_{40}H_{50}O_2$	(Diketone)

Tables 5 and 6, pp. 29–31, contain carotenoids whose structure has been established, with their order of adsorption. Similar adsorption relations are found among certain artificial degradation products of the carotenoids (Table 7, p. 32).

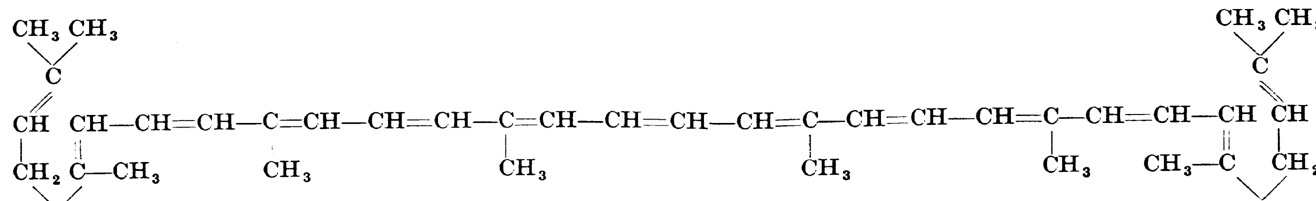
3. Aromatic Hydrocarbons with Condensed Rings

In this sphere interesting relationships between molecular structure and order of adsorption have also been established ; these are outlined below, on the basis of the work of Winter-

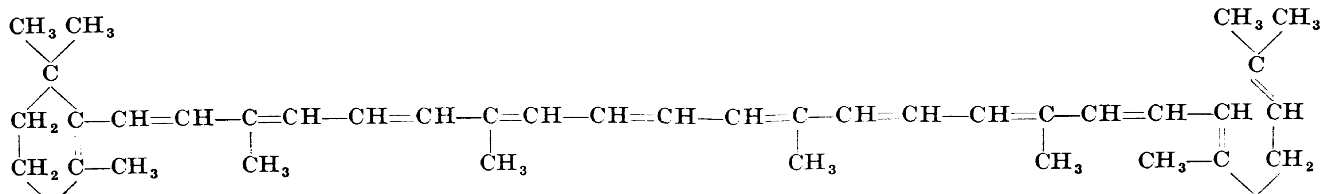
TABLE 5

ORDER OF ADSORPTION OF NATURAL POLYENE HYDROCARBONS, IN RELATION TO THEIR MOLECULAR STRUCTURE

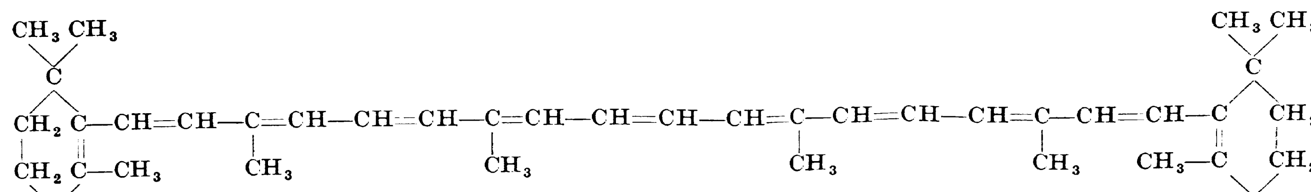
Adsorption affinity falls



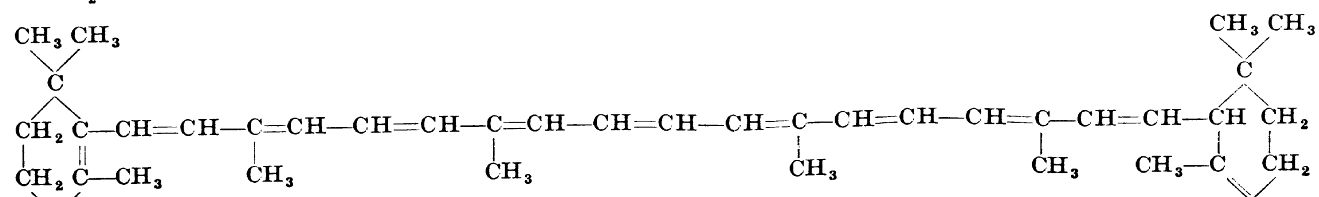
Top : Lycopene (11 conjugated and 2 isolated F).



γ-carotene (11 conjugated + 1 isolated F).

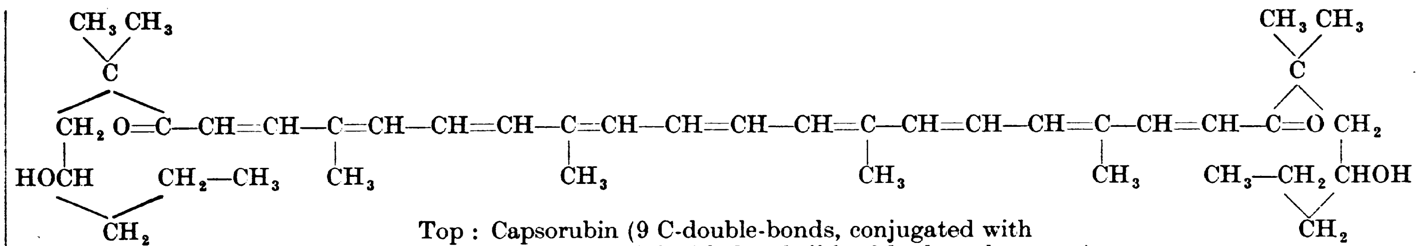


β-carotene (11 conjugated F).

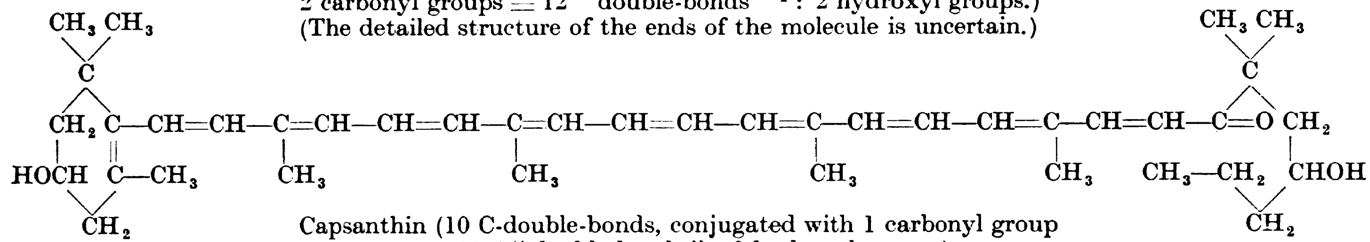


Bottom : α-carotene (10 conjugated + 1 isolated F).

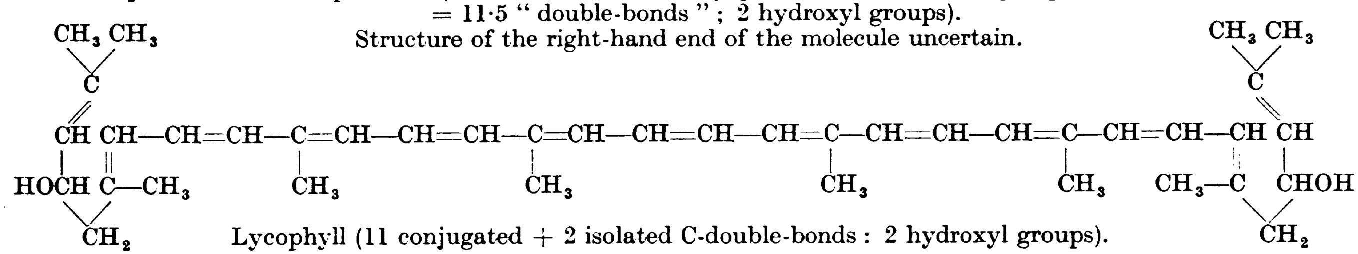
Adsorption affinity falls



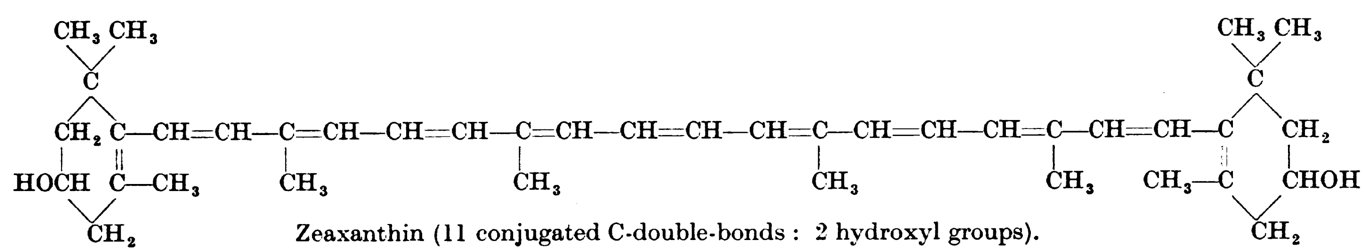
Top : Capsorubin (9 C-double-bonds, conjugated with 2 carbonyl groups = 12 " double-bonds " [1] : 2 hydroxyl groups.) (The detailed structure of the ends of the molecule is uncertain.)



Capsanthin (10 C-double-bonds, conjugated with 1 carbonyl group = 11·5 " double-bonds " ; 2 hydroxyl groups). Structure of the right-hand end of the molecule uncertain.



Lycophyll (11 conjugated + 2 isolated C-double-bonds : 2 hydroxyl groups).



Zeaxanthin (11 conjugated C-double-bonds : 2 hydroxyl groups).

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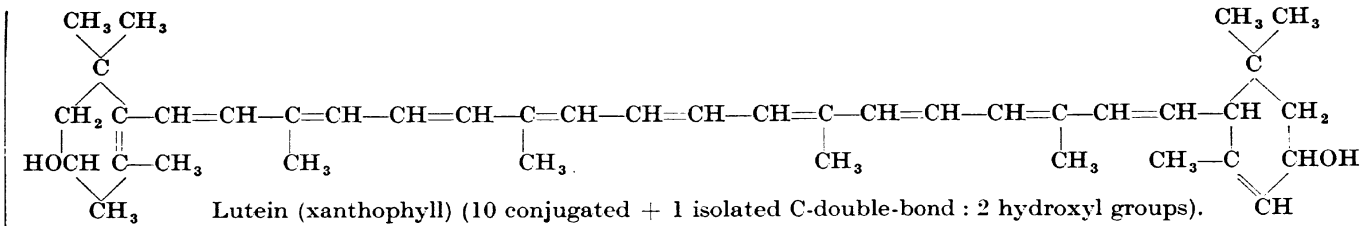
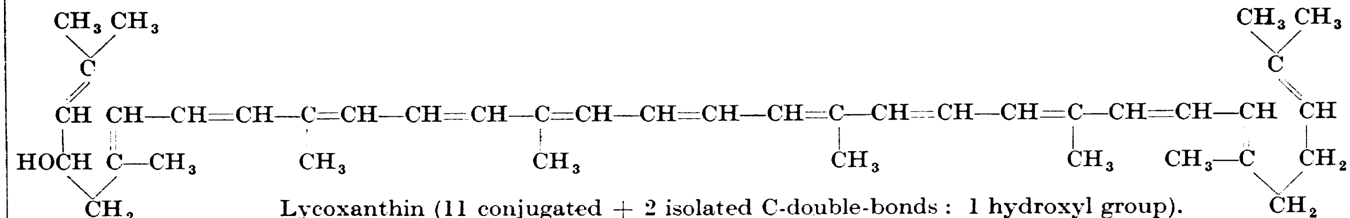
[1] A carboxyl or carbonyl group conjugated directly with an unsaturated system has an effect on colour production equivalent to that of $1\frac{1}{2}$ carbon " double-bonds," according to Kuhn and Winterstein (6). This principle is also established for adsorption affinities (see Kuhn and Grundmann, 8).

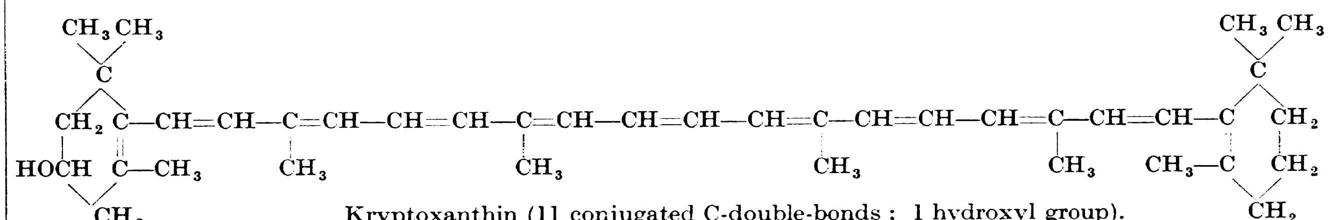
Adsorption affinity falls



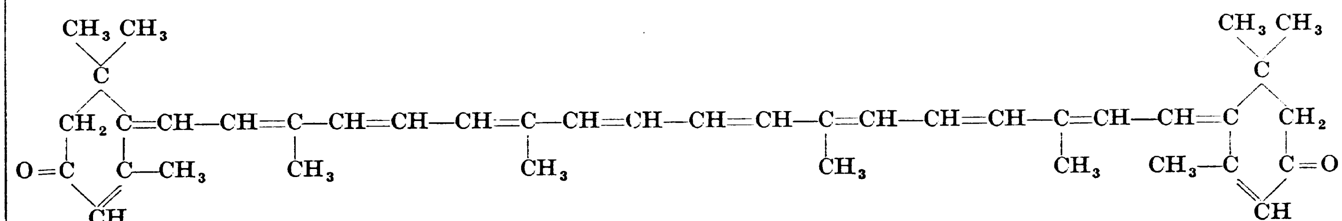
Lutein (xanthophyll) (10 conjugated + 1 isolated C-double-bond : 2 hydroxyl groups).



Lycoxanthin (11 conjugated + 2 isolated C-double-bonds : 1 hydroxyl group).



Kryptoxanthin (11 conjugated C-double-bonds : 1 hydroxyl group).

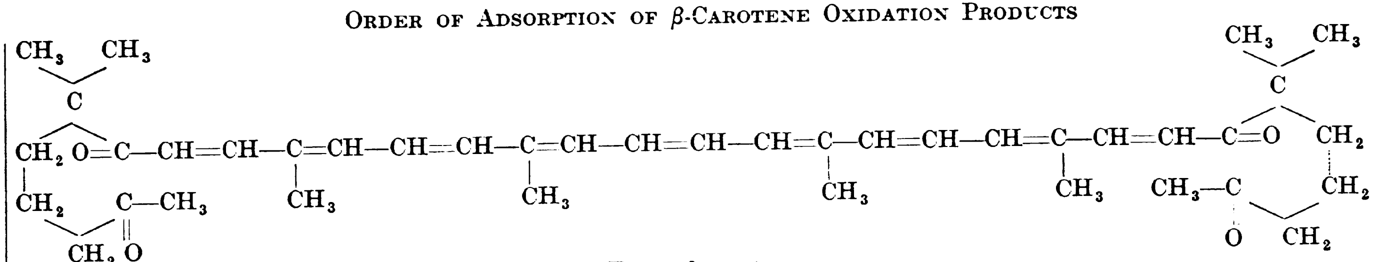


Bottom : Rhodoxanthin : (12 C-double-bonds conjugated with 2 carbonyl groups ≡ 15 " double-bonds " : no hydroxyl).

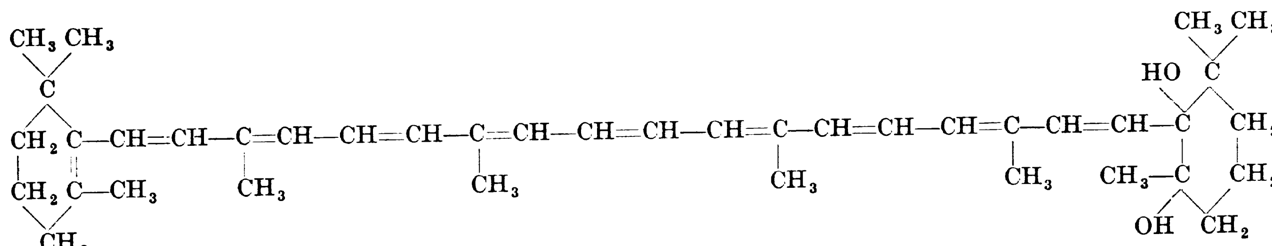
TABLE 7

ORDER OF ADSORPTION OF β-CAROTENE OXIDATION PRODUCTS

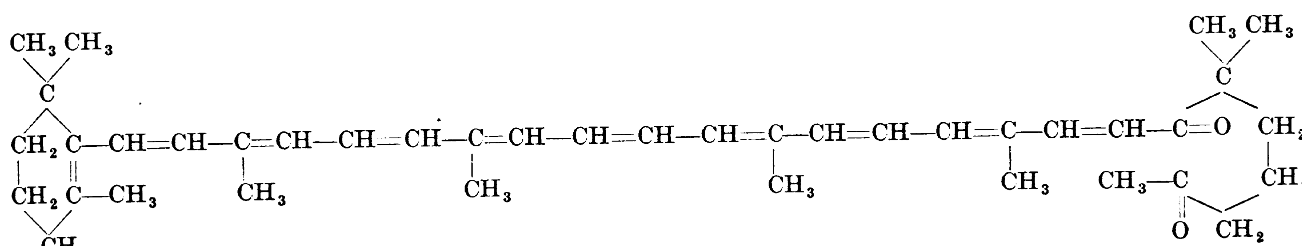
Adsorption affinity falls



Top : β-carotenone.



β-hydroxy-carotene.



Bottom : semi-β-carotenone.

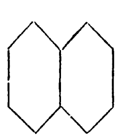
stein and Schön (2, 3) and also of Winterstein, Schön and Vetter, of Winterstein and Vetter, and of Winterstein, Vetter and Schön.

Here the same regularity is often exhibited, in that the more double-bonds are present, the higher is the relative position in the column. If the molecule consists of condensed six-membered rings only, the addition of a new ring similarly attached (with its consequent increase in carbon-content) behaves like an increase in double-bonds. Thus with substances of similar structure it is observed that the order of zone formation in the column, from above downwards, goes parallel with the diminution in number of rings, as with

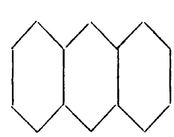
Top :	Naphthacene	$C_{18}H_{12}$: 4 rings,	9 F	(Formula III)
	Anthracene	$C_{14}H_{10}$: 3 rings,	7 F	(Formula II)
Bottom :	Naphthalene	$C_{10}H_8$: 2 rings,	5 F	(Formula I)

According to the same principle, a mixture of the four hydrocarbons below (Formulæ IV to VII) gives a succession of zones; benzpyrene is found in the upper region of the column, phenanthrene is concentrated in the filtrate, so that repetition of the adsorption procedure leads finally to an almost complete separation.

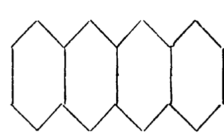
Top :	1 : 2-Benzpyrene [1]	$C_{20}H_{12}$: 5 rings,	10 F	(Formula VII)
	Chrysene	$C_{18}H_{12}$: 4 rings,	9 F	(Formula VI)
	Pyrene	$C_{16}H_{10}$: 4 rings,	8 F	(Formula V)
Bottom :	Phenanthrene	$C_{14}H_{10}$: 3 rings,	7 F	(Formula IV)



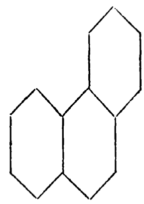
I
Naphthalene



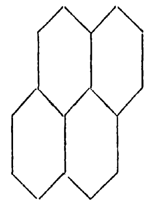
II
Anthracene



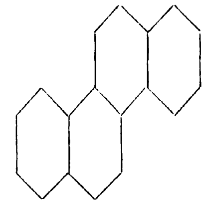
III
Naphthacene



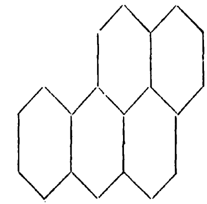
IV
Phenanthrene



V
Pyrene



VI
Chrysene

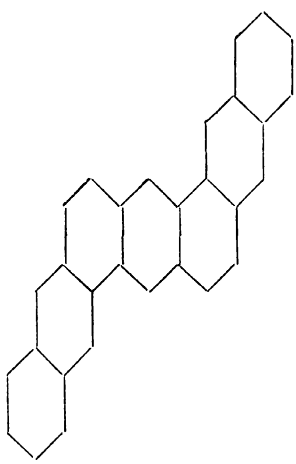


VII
1 : 2-Benzpyrene
(= 3 : 4-Benzpyrene)

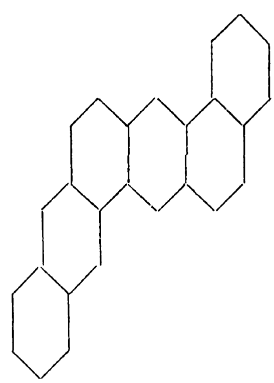
[1] Also called 3 : 4-benzpyrene.

A further example is shown by the following chromatographic series:

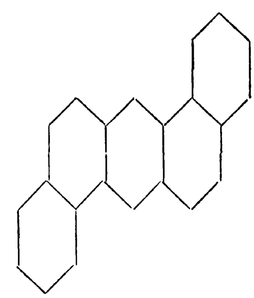
Top: 1 : 2 : 2′ : 3′-5 : 6 : 2″ : 3″-dinaphthanthracene $C_{30}H_{18}$: 7 rings, 15 F (Formula VIII)
1 : 2-Benz-5 : 6-(2′ : 3′-naphtho)-anthracene $C_{26}H_{16}$: 6 rings, 13 F (Formula IX)
Bottom: 1 : 2 : 5 : 6-dibenzanthracene $C_{22}H_{14}$: 5 rings, 11 F (Formula X)



VIII
1 : 2 : 2′ : 3′-5 : 6 : 2″ : 3″-Dinaphthanthracene



IX
1 : 2-Benz-5 : 6-(2′ : 3′-naphtho)-anthracene



X
1 : 2 : 5 : 6-Dibenz-anthracene

Impressive though this simple rule be, it is yet not sufficient to explain conditions in many instances; one has to recognise particular structural elements by which adsorbability is increased to a very marked extent, far above what is expected. One of these factors is found in the remarkable circumstances attendant on the two middle *meso* positions of anthracene: one need only recall their great potentialities for additive reactions. The "radical-like condition" present at that position is, according to Winterstein and Schön (2), associated with an increase in adsorption affinity.

Only in this way can we understand why 1 : 2-benzpyrene (5 rings, 10 F, formula VII, p. 33) lies below naphthacene (4 rings, 9 F, formula III, p. 33) in the chromatogram, for in the molecule of the former just such a *meso* position is occupied by the attached ring-system, so that the "normal" valency conditions are restored there.

We shall now turn to a discussion of the relations between

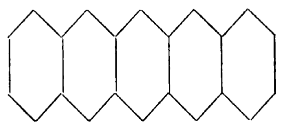
structure, adsorption affinity and colour, in so far as these have been revealed in this sphere.

The " radical-like condition " mentioned is connected both with a marked deepening of colour and with an increase in adsorbability, so that the depth of colour (i.e., tint)—as indicated by, for example, the position of the longest wave-length at which the absorption curve shows a maximum wave-length—is related with adsorption affinity. This expresses itself, as with the carotenoids, in the fact that certain more deeply coloured hydrocarbons are found higher up in the Tswett column than related compounds that absorb at shorter wave-lengths. The same is true when the extinction maxima are displaced into the ultra-violet.

Further examples :

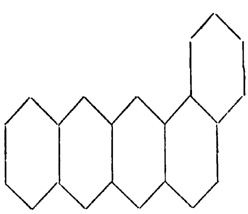
(*a*) Top : Naphthacene (orange-red, Formula III, p. 33)
Bottom : Chrysene (colourless, Formula VI, p. 33)

(*b*) Top : Naphthacene (orange-red, Formula III, p. 33)
Bottom : 1 : 2 : 6 : 7-dibenzanthracene (orange-yellow, Formula below)

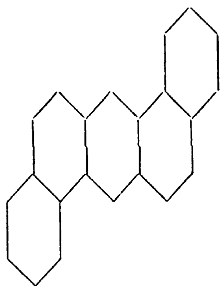


2 : 3 : 6 : 7-Dibenzanthracene

(*c*) Top : 1 : 2 : 6 : 7-dibenzanthracene (orange-yellow, Formula below)
Bottom : 1 : 2 : 5 : 6-dibenzanthracene (colourless, Formula below)



1 : 2 : 6 : 7-Dibenzanthracene



1 : 2 : 5 : 6-Dibenzanthracene

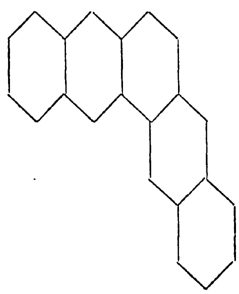
As an illustration, take the example of the orange-red naphthacene (Formula III, p. 33). According to an hypothesis put forward by Clar (and others, not acknowledged by Scholl), the radical-like condition of anthracene is appreciably increased by the linear attachment of a further benzene nucleus. Accordingly there is also an increase of adsorption

affinity on the column, so that naphthacene (2 : 3-benzanthracene) is more firmly bound than any other four-ringed hydrocarbon of the series. Furthermore, among the pentacyclic compounds, 2 : 3 : 6 : 7-dibenzanthracene, which has a similar linear structure, takes a higher place than naphthacene.

The difference in adsorption behaviour of the last two isomers must be conditioned by the fact that in one compound four rings are in linear connection, in the other only three. For Clar points out that an " angle " connection lessens the " anormal " condition, as well as the depth of colour.

A typical result is also obtained in the following separation :

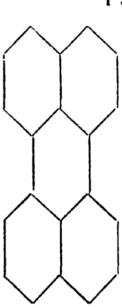
(*d*) Top : 1 : 2 : 6 : 7-dibenzanthracene (orange-yellow) } see Formulæ
1 : 2-(2′ : 3′-naphtho)-anthracene (pale yellow) }



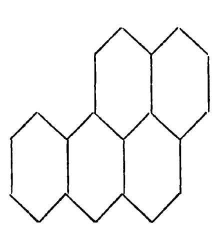
2 : 3-(1′ : 2′-Naphtho)-anthracene

It should be noted that chromatography of mixtures (*c*) and (*d*) must be repeated several times, as the differences in adsorption are actually slight. If another benzene ring is introduced into each of the last-mentioned pair, the separation fails altogether.

(*e*) Top : Perylene (orange-yellow) } see Formulæ
Bottom : 1 : 2-Benzpyrene (pale-yellow) }



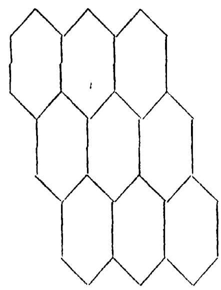
Perylene



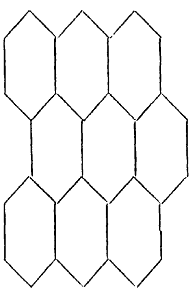
1 : 2-Benzpyrene (= 3 : 4-Benzpyrene)

Perylene and 1 : 2-benzpyrene are isomers ($C_{20}H_{12}$). They

are pentacyclic and without free *meso* positions: the reason for their successful separation is as yet not clear. Adsorption affinity in the class of compounds in question is strongly affected even by slight differences in structure, as is established by the fact that anti-diperi-dibenzcoronene can be separated chromatographically from anthrodianthrene.



Anti-diperi-dibenzcoronene



Anthrodianthrene

To sum up—the investigations of Winterstein on the one hand mark out the limits of application of Tswett's procedure to a sharply defined class of substances and on the other hand make clear that both increase in colour and rise in adsorbability originate in the same structural cause.

The enumeration of these particular compounds containing carbon double-bonds merely constitutes a special case, particularly easy to establish, of a more general law.

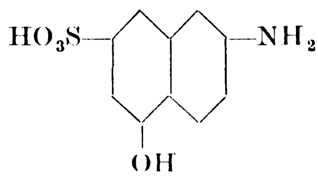
4. Azo Dyestuffs

In view of the extraordinarily large number of substances belonging to this class, its clarification by adsorption analysis will offer opportunities for more extended research. Interesting results have already been obtained by Ruggli and Jensen (1, 2) and the following examples are taken from their work (for individual instances, see also p. 190 and particularly Jensen).

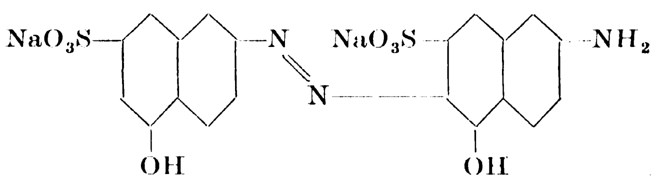
If the rest of the structure is laid down in general terms, adsorption affinity increases parallel with the number of azo-groups: thus, for example, the following order of zoning has been established (aqueous solution, column of Al_2O_3—as also in the other separations mentioned).

Most strongly adsorbed: Diamine Green (trisazo dye)
↓ Congo Red, pure (bisazo dye)
Most weakly adsorbed: Diamine Rose FFB (monoazo dye)

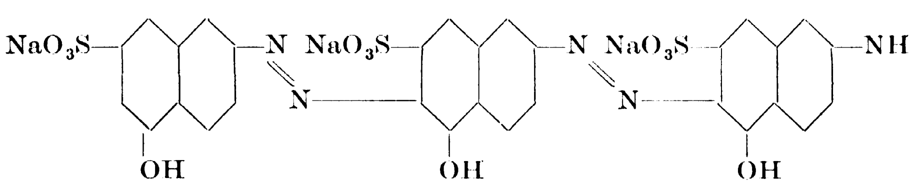
This influence is very prettily demonstrated in that series of homologous azo compounds in which the representatives succeed one another by stepwise diazotisation and alkaline coupling with the so-called "J-acid" (2-amino-5-naphthol-7-sulphonic acid), and the dyestuffs derived from them :



J-Acid



2 J-Acid (diazotised J-Acid coupled with J-Acid)



3 J-Acid
and so on.

Dyes constituted according to the scheme

$$\text{J-acid}-(\text{N}=\text{N}-\text{J-acid})_n$$

arrange themselves in the following order (0·8 g. in 250 ml. water, developed with 750 ml.) :

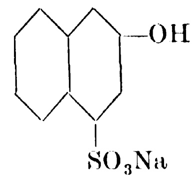
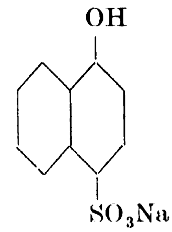
Most strongly adsorbed $n = 4$, dark violet zone
↓ $n = 3$, light violet zone
$n = 2$, reddish violet zone
Most weakly adsorbed $n = 1$, orange-red filtrate

A dye of unknown structure (value of n unknown), that arises by self-coupling, appears below the place of $n = 2$.

With an identical number of azo-groups, other factors, according to Ruggli and Jensen (1, 2), have been found appreciably to influence adsorption affinity :

(*a*) A hydroxyl group in the β-position of the naphthalene nucleus appears to be more powerful than an α-hydroxyl group ; thus the sodium salt of 2-naphthol-4-sulphonic acid

appears higher in the chromatogram than that of 1-naphthol-4-sulphonic acid

Top   Bottom

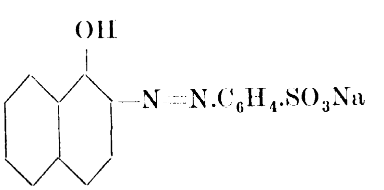
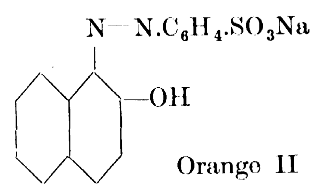
(*b*) An amino-group in the β-position produces stronger adsorption than one in the α-position in structures otherwise analogous ; thus :

Most strongly adsorbed	Product of coupling diazotised benzidine with two molecules	2-amino-8-naphthol-6-sulphonic acid
↓		2-amino-5-naphthol-7-sulphonic acid
		6-amino-2-naphthol-4-sulphonic acid
		1-amino-8-naphthol-4-sulphonic acid
Most weakly adsorbed		1-amino-5-naphthol-7-sulphonic acid

(The last two dyes are adsorbed about equally.)

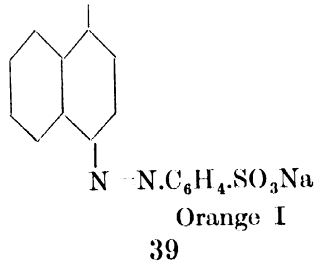
All these relationships naturally apply only to aqueous solutions and alumina. If the sulphonic groups are removed and a non-aqueous medium is used, the adsorptive powers very often bring about quite a different order of arrangement.

(*c*) *o*-Hydroxy-azo dyes are better adsorbed than *p*-hydroxy-azo dyes. This powerful influence has an equalising effect in the following pair, which are equally strongly bound, in spite of the difference in position of the β- and α-hydroxyl groups.

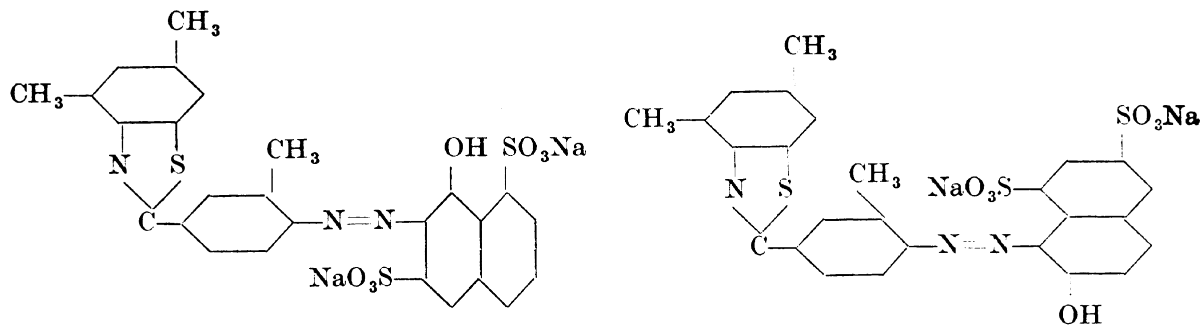
 

Orange II

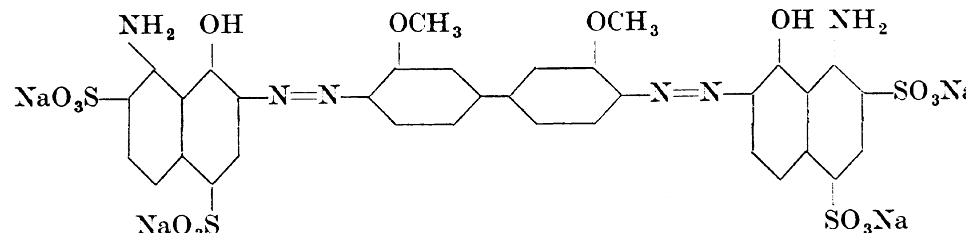
Orange I, however, is retained with appreciably less effect, owing to the coupling in the *p*-position.



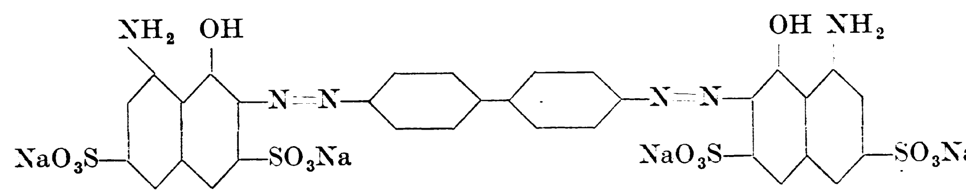
Orange I



Erika B (above) chromatographically not separable from Erika G extra (above)



Direct Sky Blue green shade separable from Direct Blue 2B (below)



Direct Blue 2B

(*d*) The following example demonstrates how difficult it is to forecast the behaviour of mixed dyes in the column and the consequent ease of separation of one representative from others. Erika B and Erika G extra cannot be separated by adsorption analysis, whereas Direct Sky Blue green shade and Direct Blue 2B are easily separated, although the structural differences are little greater than in the first-mentioned pair. Perhaps the methoxyl groups are here responsible.

5. The Fluorescein Group

According to Ruggli and Jensen (1), there is here a marked dependance of behaviour on halogen content, the adsorption affinities increasing with the number and atomic weight of the halogen atoms. As increasing depth of colour is caused by the same factors, there is here also to be seen a parallelism between depth of colour and position in the chromatogram (water and alumina):

Most strongly adsorbed	Rose Bengal (4I, 2Cl)
	Phloxin (4Br, 4Cl) and Erythrosin (4I)
↓	Spirit Eosin (Eosin methyl ester, 4Br) and Eosin (4Br)
Most weakly adsorbed	Fluorescein (halogen-free)

CHAPTER 2

METHODS

Adsorption Media

General

In theory all substances in powder or finely divided form can act as adsorbents, as also can fibres, provided always that they are not soluble in the solvent used and that they have no destructive action on the compound to be adsorbed. Tswett (1) himself had already tested over one hundred adsorption media.

Nevertheless it has become evident that there is generally a much narrower choice. First are eliminated most organic materials, for practical reasons ; they can only be dried with care and cannot be regenerated by ignition. Moreover, such substances are frequently accompanied by small quantities of soluble matter, which have to be removed by a troublesome preliminary extraction. Of this class of material, inulin and powdered sucrose have, so far, been tested and both were recommended by Tswett (1) ; lactose has also been used. The number of organic adsorbents should, however, increase again, for many possibilities have not yet been explored : thus, Ruggli and Jensen (1) report experiments in chromatography with cotton-wool.

In the realm of inorganic adsorbents, strongly acid or strongly basic compounds can be ruled out ; as a rule so can dark or strongly coloured substances, for the sequence of layers on them would be difficult to establish. If, however, the individual components are to be eluted directly from the undivided column, then the last limitation does not apply.

The relationship between the quantities of adsorbent and adsorbed material shows extreme diversity in the different

types of experiment and varies from 1 : 10 to 1 : 100,000. Any appreciable wastage of the column's material is an especial disadvantage on a large scale.

Selection of the adsorbents has to be made empirically, although there is already to be found in the literature quite an amount of information that can serve as indications for different classes of compound. The examples given below apply to temperate climates, for adsorption relationships may in some instances be quite different under tropical conditions. Thus van Veen and Lanzing found on investigating the polyenes of blood-serum that lime and alumina adsorb noticeably less strongly in Batavia than in Europe. The former adsorbent was, in fact, valueless, while the latter was useful and differentiated well.

Naturally it is not only the chemical nature of the compound that is of importance, but also, and to a very marked extent, its quality. As is well known, preparations having the same chemical name, but of different origins, often behave quite differently in adsorption experiments, both quantitatively and qualitatively. Above all, the extent and the nature of the individual particle surfaces are determining influences. There are also unknown factors that favour the use of a particular preparation, one, for example, having a particular trade name. The presence of chemical impurities is not an invariable disadvantage : it can even exert a favourable influence (*v.* p. 21).

Strong adsorbents are called "active" ; activation and de-activation can be deliberately undertaken (p. 48).

For the particular purposes of chromatography, filling the tube with material of maximum activity is not always effective, compared with certain earlier adsorption methods and with certain modern procedures for purification, especially on a manufacturing scale. If the solid phase acts too energetically, then it takes up indiscriminately all substances in the solution and binds them firmly ; it is just this firmness of attachment that evens out the smaller differences between individual adsorption affinities and conceals them. The constituents remain undifferentiated and do not form clear-cut zones even on development. The replacement phenomena described on page 17 do not come sufficiently into play, for the countless

simultaneous single elutions meet with opposition. Similarly elution of the portions of the column at the end of the experiment can become difficult or impossible.

In working with Tswett's technique, we require to follow a middle course in our demands of the adsorbent : the components must be firmly held, yet in a condition to travel downwards with moderate speed during the washing process and thus to be separated from one another. In such circumstances elution of the individual compounds follows a satisfactory course.

It is, of course, also possible so to conduct the experiment that different adsorbents are used successively in separate charges. For example, the total pigment in a mixture can be held on a column to separate colourless impurities, and then submitted to finer differentiation by use of a less strongly adsorbent material as a second charge.

PARTICLE SIZE. We shall now indicate certain granule sizes, that derive partly from the material customarily used in the authors' laboratory (Table 8). The figures are of use only as a general guide. They indicate the mean granule size of the mass, but there may be present particles showing very marked departure from the mean.

The particle size of the column contents must generally not be too diverse, otherwise the finer particles may act almost like a different adsorbent and work too vigorously. In one exceptional instance, however, Koschara (4) found that a pigment was in fact adsorbed with particular tenacity (irreversibly) by the coarser particles of alumina. Here we have an approximation to a chemical difference between the largest and the smallest particles.

The range of utility of some adsorbents can be very nicely graded by using various proportions of a coarser and a finer variety. A mixture of precipitated calcium carbonate (Merck) and the light calcium carbonate of pharmacy, in the ratio of 20 : 1, is very suitable for working up certain polyene alcohols, but it is insufficiently active to bind the wax pigments (esterified polyene alcohols). For this purpose the proportions should be between 5 to 1 and 4 to 1. With a ratio of 1 : 1 even lycopene is adsorbed from petroleum, although otherwise it passes through a calcium carbonate column. For fixing

carotene the last-named mixture is too inactive and useless (Zechmeister and Cholnoky, 2).

TABLE 8

AVERAGE PARTICLE SIZE OF CERTAIN ADSORBENTS

Adsorbent	Particle Size (μ)
Alumina (Merck), standardised by Brockmann's method	7
Commercial alumina, produced in the laboratory	2
Acid Clay (Java) *	10
Precipitated calcium carbonate (Merck)	1·5
Light calcium carbonate (commercial)	1·2
Calcium Hydroxide, prepared in the laboratory	2·5
Gypsum (hydrated) †	10·5
Magnesium oxide ‡	1·5
Bleaching earth	3
Floridin	1·5 to 7
Floridin XXF	1·5 to 6

* Particulars from Euler and Gard. This clay is a calcium-aluminium-magnesium silicate. The surface of 1 g. is of the order of 10 square metres and is twice as great as that of the powdered silica used by Bowden and Bastow.

† According to Karrer and Weber.

‡ Also contains tile-shaped crystals.

Very different demands are made of an adsorbent, according as it is to be used in an anhydrous or an aqueous medium. In the first instance any moisture content of the column contents is generally harmful and must be eliminated by heat and the adsorbent must subsequently be stored out of contact with air ; such pre-treatment can also influence its activity. If the experiment is carried out with moist or aqueous solutions, then the adsorbent must not fix water, must not swell, and so on, for then gaps arise in the column, besides which the flow may be impeded. Pressing out the column is also rendered more difficult thereby. For this reason lime, otherwise much used, is unsuitable in such circumstances. Again, one can use the device of filling the tube with, for example, hydrated gypsum (Karrer and Weber, p. 180).

It is easily understood that in the adsorption analysis of aqueous solutions there were first brought into use those

commercial products that have for a long time been employed industrially for purification and decolorising processes, such as bleaching earths.

Special Data

Below are given certain particulars of the most important adsorbents currently in use.

POWDERED SUCROSE had already been used by Tswett (1) for the separation of chlorophyll. There are some sugars that adsorb more strongly, and some, such as lactose, more weakly (Winterstein and Stein, 2). For lactose, see also page 23.

INULIN is less often used (p. 90) : its laboratory preparation has been described by, for example, Spoehr.

ALUMINA (also hydrated) is perhaps the most widely used of adsorbents, and is very serviceable for aqueous as well as for anhydrous media. Preparations made by various procedures and also commercial brands show great variations in adsorptive power, so that the quality of the material should always be clearly defined.

The " fibrous alumina " of Wislicenus (Merck, prepared from the amalgam) is very powerful undiluted, but still active when mixed with other varieties ; it is much too expensive for large-scale experiments.

PREPARATION OF FIBROUS ALUMINA BY THE METHOD OF WISLICENUS.

200 g. of aluminium filings of uniform particle size—0·5 mm.—are treated in an enamelled jar (*c.* 15 × 15 cm.) with 200 ml. of 10 per cent sodium hydroxide solution, until hydrogen is rapidly evolved. The resultant grey foamy slime is rinsed away several times with a strong stream of water, and the reaction is repeated with a further 100 ml. of sodium hydroxide solution until hydrogen is again vigorously evolved. After several rinsings, continued until the wash-water is clear, 50 g. (about 40 ml.) of cold saturated mercuric chloride solution are added, the resultant dark slime being kept vigorously agitated. This slime is washed off with a strong stream of water and the water is poured off until it drains in single drops. A mixture of 2 to 2·5 g. of nitrobenzene, 18 to 20 g. of ether, 20 g. of 90 per

cent alcohol and 10 to 12 g. of water are added, with agitation: the mixture develops considerable heat and is allowed to stand for some hours. The distended yellowish mass is floated off with alcohol from unused aluminium, allowed to dry partially, filtered by suction, and heated to redness, after sufficient has been collected, in a flat fire-clay dish in the muffle. Finally the white mass is freed of the finer dust and the coarser granules, as well as of particles of aluminium, either by flotation with alcohol or ether by means of a current of dry air, or in a long wide tube, fitted to two large receiving flasks. The material should be kept in a desiccator or a well-stoppered flask and 1 g. should take up 0·33 to 0·4 g. of tannin when it is added gradually to 200 ml. of a 0·3 to 0·4 per cent solution of pure tannin. Renz gives the following directions for the preparation of fibrous alumina: aluminium filings are treated in a porcelain basin with a little water and a few millilitres of freshly prepared mercuric acetate solution. A reaction sets in immediately, with evolution of a considerable amount of heat. A little water is added very slowly until the reaction is ended and the alumina is obtained in the form of a flocculent light grey powder.

MERCK'S ALUMINA—standardised according to Brockmann's method—is characterised by a very welcome constancy in its properties: it is adjusted to a definite moisture content by heat (alleged to be applied in a stream of carbon dioxide) and prepared at the required degree of activity with the aid of suitable pigments. This granular product is, however, expensive and is frequently used in admixture with the ordinary commercial product, particularly as for certain purposes it adsorbs too strongly. It should be noted also that standardised alumina prepared by the method of Cahn and Phipers has a more strongly alkaline reaction than the ordinary kinds; this calls for special care in its use for the treatment of alkali-sensitive substances.

For many purposes ALUMINIUM OXIDE-HYDRATE (or aluminium oxydatum anhydricum) will be found not only valuable but also good value. Aluminium hydroxide, $Al(OH)_3$, can also be dehydrated in the laboratory, yielding a strongly active product. Hesse (private communication) ignites 200 to 300 g. of technical aluminium hydroxide at a time (Merck or Kahlbaum) in an enamelled pot over a powerful

burner until no more spurting occurs. Chemically pure alumina has no advantages.

"HYDRALO" is an American drying agent recommended by Strain and stated to have a more powerful action than fibrous alumina (Aluminium Co. of America).

Holmes, Lava, Delfs and Cassidy have examined exhaustively the activity of preparations made in various ways; some of them are commercial products. The adsorbents were mostly passed through a 200-mesh sieve and activated by heating for 2 hours at 200° C. in carbon dioxide, to secure removal of air and water.

Examples. (*a*) A commercial product was washed until free of alkali and then activated.

(*b*) Aluminium nitrate containing water of crystallisation was kept between 200° and 300° C. until (after some hours) no more nitrous fumes were evolved.

(*c*) Aluminium chloride was treated with a small excess of ammonium hydroxide; the solution was allowed to evaporate over a period of two or three months; the residue was washed free of chloride, dried and sifted.

(*d*) Aluminium ethylate was spread on glass and kept for 5 weeks in a warm damp atmosphere, with occasional kneading; it was next heated to 210° C. for 3 hours in a stream of dry carbon dioxide until no more moisture is liberated; it was then powdered and activated once more.

(*e*) Preparations were also made on pumice, particularly by slow hydrolysis of aluminium alcoholates.

Procedures of this kind are too tedious for the purposes of practical chromatography.

Ruggli and Jensen (1, 2) have contributed appreciably to progress by the discovery of a simple device for activation: it consists in rinsing the oxide with tap water and then heating strongly again. For most purposes a single treatment suffices; if this should prove insufficient for the particular purpose involved, it can be repeated as often as necessary. Owing to the varying hardness of different water samples, Franck carries out this activation with 5 per cent calcium hydroxide solution. As rinsing with distilled water is ineffective, it is apparently a matter of charging the adsorbent with a trace of lime. The procedure has the advantage that the effectiveness of the

column contents can be thereby graded; thus Karrer and Strong found that alumina that had been only slightly activated gave good results in the chromatography of the anthocyanins.

It sometimes happens, though less often, that the surface of a sample of alumina acts too strongly and must be de-activated. According to Heilbron and Phipers this is achieved by taking, for example, the standardised "Brockmann" alumina of commerce, washing it with methanol and drying it in air.

Methods of preparing oxides and hydroxides of trustworthy activity are still deserving of further development, on the industrial scale also.

Finally, it should be noted that alumina columns must be extruded in a moist condition, as they are otherwise easily broken; this is especially so with the standardised product.[1]

MAGNESIUM OXIDE has been recommended by, among others, Euler and Gard and especially by Strain (1 to 5). It acts with very varying vigour, according to the method of preparation. If the metal is simply burnt, the resulting oxide has only a trace of adsorptive activity. The product obtained by heating the carbonate at relatively high temperatures has also only moderate activity. Much better results are achieved by dehydration of magnesium hydroxide at not too high a temperature. A commercial American product (Micron Brand Magnesium Oxide, no. 2641, California Chemical Corporation, Newark) is recommended. Our experience indicates that the ordinary commercial variety is adequate for most purposes. For other magnesium compounds as adsorbents, see Euler and Gard.

CALCIUM HYDROXIDE. According to Karrer and Walker (1) commercial slaked lime makes an admirable adsorption column and possesses the advantage of extreme cheapness, as does also calcium oxide, which is occasionally used. For the analysis of solutions containing water it is naturally useless—the tube becomes blocked or, if slaking was incomplete, the whole mass swells up. In our laboratory commercial quicklime is sprinkled with only sufficient water to cause it to fall to powder. It is sifted through a 120 or a 180-mesh sieve, according to the degree of dispersion required. It is hygroscopic and must be protected

[1] See translators' note at end of General Section, Chapter 2, p. 88.

from air ; before use, the containing vessel must be well shaken, in order to distribute uniformly the upper layers, which have an enhanced carbonate and moisture content.

CALCIUM SULPHATE (hydrated) is used by Karrer and Weber in an aqueous medium (p. 180).

CALCIUM CARBONATE was frequently used by Tswett ; it is a mild adsorbent, widely applicable. Information has already been given (p. 44, above) about the relation between the particle size of calcium carbonate and its activity. By appropriate blending of coarse and fine powders it is possible to adjust adsorption activity to particular requirements. Moist preparations work badly and must be heated at 150° for some hours.

Note to 1st English Edition. Soda-ash has been recommended for separating chlorophyll from carotenoids.—TRANSLATORS.

BLEACHING EARTHS are hydrated aluminium silicates, used in technology, with widely varying calcium, magnesium and iron contents, and having clay-like properties. A distinction must be made between natural earths, that have only undergone mechanical processing, and activated earths. On a manufacturing scale activation consists for the most part in treatment with hydrochloric acid ; this dissolves certain constituents. The surface condition (porosity) is thereby rendered more favourable ; further, such preparations possess acidic groups and have thus the capacity to form salts during the course of adsorption.

According to the investigation of Carlsohn and Müller (1, 2), this is nothing to do with the presence of traces of mineral acids ; thus, from benzene solution, Sudan Red—the product obtained by coupling β-naphthol with diazotised *o*-aminoazotoluene—gives a blue adsorbate on alumina, and similarly on kaolin previously treated with acid, but on activated bleaching earths a violet-blue to greenish-blue adsorbate, due to formation of salts. After complete elimination of the loosely bound water from the earth, by means of moderate heat, the shade of the Sudan Red adsorbate approximates to the green tint seen in sulphuric acid solutions of the dye.

For the treatment of aqueous liquids, Koschara (4) has had good results with the following adsorbents :

FRANKONITE KL : activated with hydrochloric acid, acid to Congo Red (Pfirschinger Mineralwerke, Kitzingen).

FLORIDIN XXF : finely divided, very active even though not pre-treated : pale grey (H. Bensmann, Bremen).

FLORIDIN XS : similar, but coarser and sandy.

The second (floridin XXF) preparation was found by Schöpf and Becker, when investigating the pterins, to be too active ; it adsorbed erythropterin, for example, irreversibly. Probably the iron content is involved in this, for the adsorbate shows the same colours as those obtained with acid solutions of erythropterin and ferric chloride. When the iron content of the floridin was markedly lowered by boiling the earth for 3 to 5 minutes with 10 parts of 3N-hydrochloric acid, the material showed just the right adsorptive power for the investigation in question. If floridin prepared in this way is boiled with further acid, the ability to take up the pigment disappears along with the iron content.

Often assessment of a chromatogram is upset by the dark colour of the damp bleaching earth itself ; in some such instances a better picture can be obtained by pressing out the adsorbent and drying it or by using ultra-violet light.

FULLER'S EARTH, of various origin and quality, is also used. It generally has a distinct acid reaction and is more or less dark in colour, especially when moistened with liquid. " Lloyd's Reagent " is a frequently used variety.

Artificially prepared ZEOLITES have so far been little employed ; Cerecedo and Hennessy use the zeolite " Decalso " (Permutit Co., New York).

LESS FREQUENTLY USED INORGANIC ADSORBENTS. It is not proposed here to enumerate all of these. In some instances difficultly accessible adsorbents are used ; they can easily be replaced by those more generally available.

Occasionally the following have been found of value : sodium sulphate (anhydrous), lead sulphide, talc, kieselguhr, infusorial earths, kaolin, silica gel (non-elastic, e.g., the " intermediate activated " variety of the Silica Gel Corporation, Baltimore), also " Tonsil " and " Clarit " (Moosburg clays). Some charcoal preparations (Vereinigte Farbwerke, Werk Düsseldorf ; Anticarbone, Paris) are strongly adsorbent and easily accessible, though they involve foregoing a visible

chromatogram and carrying out fractional elution or an empirical division of the column. "Carboraffin" (obtained by carbonisation of pine-wood with zinc chloride) and "Norit" (a variety of wood-charcoal activated with super-heated steam) are also to be recommended ; they can be de-aerated by heat in a stream of nitrogen. Powdered blood-charcoal can sometimes be used.

When technical products are used, it is necessary to determine by trial whether they yield material to the solvent.

Regeneration of used column contents is seldom worth while on the small scale. Inorganic substances can be thoroughly washed with a suitable solvent and recovered after ignition, provided they are not loaded with carbon compounds that resist carbonisation. It is necessary also to establish that there has been no loss of adsorptive power. Used calcium carbonate can often be recovered by extraction with cold or warm methanol, followed by air drying ; it is then essential to maintain the material for a considerable time at 100° to 150° C. For technical regeneration of bleaching earth, especially those containing fatty matter, see Eckart.

Below are given methods for simultaneous use of two absorbents in the same tube, often to be recommended. Either they can be used together as a homogeneous mixture or the column can be built up partly from one and partly from the other, each being pressed in separately.

When use is made of a uniform mixture, the object is generally to secure some intermediate degree of adsorptive power or else to increase the speed at which the liquid passes through, by admixture of a coarser material. It should be noted that development or partial elution may upset a fractionation if the two adsorbents have too widely different actions.

To increase the speed of flow it sometimes helps matters to mix the adsorbent with sand or powdered glass.

For demonstration purposes or to save labour, adsorbents in different layers may be put in the same tube. The upper half of the column is built of a weak, the lower of a more powerful adsorbent. The contents of the intermediate filtrate pass into the lower part of the tube and are held there, thus obviating the preparation of a second column. Examples of this procedure are :

(*a*) Column of calcium carbonate and alumina, for microanalysis of polyene extracts ; Kuhn and Brockmann, 3 (Fig. 14, p. 61).

(*b*) Column of calcium carbonate and calcium hydroxide, for estimation of paprika pigments ; Cholnoky, 3 (p. 133).

(*c*) For working up polycyclic hydrocarbons, Winterstein, Schön and Vetter placed above the alumina column a thin layer of mixed alumina and "Carboraffin," which retained certain impurities (p. 211).

(*d*) Three adsorption layers, sucrose above, calcium carbonate in the middle, alumina below, can be used to demonstrate the components of leaf green ; Winterstein (1) (Fig. 36, p. 92).

Solvents

Operations conducted in the absence of water will be discussed first : the use of aqueous solutions will be considered on page 77.

Choice of solvent naturally depends first of all on the solubility relationships of the substance. If this is already in solution, for example as an extract, it is dissolved in a suitable medium after concentration under reduced pressure. When working with anhydrous liquids, it is generally desirable to secure complete absence of moisture, if necessary by appropriate preparatory treatment of the solvent. The inclusion of solvents that are immiscible with water seems also an advantage. It is a drawback of the Tswett method—as also, however, of some other adsorption procedures—that the solutions must generally be very dilute. Crystallisation in the column must be avoided at all costs ; consequently the material to be adsorbed should seldom be more than a few per cent of the crude solution. An impurity, provided it exerts no disturbing influence in other ways, may of course be present at a higher concentration.

Theoretically every liquid is an adsorption medium as well as an elution medium ; what part it is considered to play in the chromatogram depends in each instance on the course of the adsorption isotherm. The principles that apply here are

similar to those that determine the choice of adsorbent. If the substance is too strongly held, difficulties arise in development and in the final elution ; if, on the other hand, the adsorption affinities operating in the system are too weak, the substance runs quickly through the column, before a satisfactory array of zones has been built up. (Sometimes this is intentionally arranged ; "liquid chromatogram," p. 76). As can be understood, various mixtures of two solvents can be chosen to direct the experiment along the right course.

Often the solvents enumerated below will be found sufficient. As in other methods of purification, liquids of high boiling-point are useless, if a labile substance is finally to be isolated. Too low a boiling-point is undesirable because it involves excessive loss by evaporation. Boiling-points between 40° and 80° C. are the most suitable.

CARBON DISULPHIDE (b.p., 46° C.) is in many instances very successful, for example, with calcium carbonate. The technical grade may have to be purified. Continued operation with this solvent is unpleasant and injurious to health, especially if the experimenter has many columns to cut. In that event, ventilation should be provided. Further, the artifice may be used of replacing the carbon disulphide in the finished chromatogram with petroleum, before pressing out the column.

LIGHT PETROLEUM (petroleum spirit, ligroin, petroleum ether, benzine ; b.p., not above 80° C.) is at present the most frequently used solvent, because of its purity, cheapness and other good qualities. The use of alumina or calcium hydroxide with light petroleum has often given excellent results. Pentane (b.p., 36° C.) can also be used.

BENZENE (b.p., 80·4° C.) is also extensively employed. It differs from light petroleum in that in many instances it has a stronger eluent action. From this the use of mixtures of benzene and light petroleum follows naturally. Thus adsorption may be from petroleum and development with petroleum and benzene ; again, adsorption may be from a 10 to 1 (petroleum to benzene) mixture and development with a 3 to 1 mixture. In some circumstances pure benzene can be used as eluent.

Less frequent use is made of chloroform, carbon tetrachloride, methylene chloride, ethanol, anisole, acetone, ether

and so on. The last-named is generally used for elution ; its action is partly due to its alcohol content. In any event the ether must be peroxide-free ; as is well known, this is achieved by keeping it for some days over aqueous ferrous sulphate solution.

Eluents

Tswett established many years ago that numerous adsorbates are rapidly broken up by methanol, ethanol or acetone. On this observation is based the use of one of the best and most accessible of eluents. It is generally not necessary to use pure alcohol, but is quite sufficient to add from 0·5 to 2 per cent to the solvent that has actually been used : elution can also be carried out by means of ether containing a small amount of methanol.

If a chromatographic experiment has to be repeated, either with a portion of a column or with the whole contents, it is, for example, possible to elute with petroleum containing a small amount of wood spirit ; the petroleum solution is then filtered free of adsorbent, washed free of alcohol with water, dried and poured on to a fresh column, the need to concentrate the solution being thus avoided.

Lower alcohols do not always lower or raise the adsorptive forces, as is shown in the investigations of the pterins by Schöpf and Becker (*v.* p. 169) ; they carried out adsorption from methanol. For adsorptions from ethanol, see, for example, Kuhn, Desnuelle and Weygand (p. 206).

In certain instances elution may be very difficult to effect, and boiling with a lower alcohol may be tried. Cold pyridine has often proved useful, alone or in admixture with, for example, water or ether. It sometimes happens, though rarely, that none of the chosen methods avails to break up the adsorbate. With acid-resisting substances recourse is then had to emergency methods and the calcium carbonate, for example, is dissolved in dilute hydrochloric acid, thus permitting extraction of organic substances ; columns of sucrose are dissolved in water.

Occasionally one encounters a substance that is not adsorbed at all by calcium carbonate and is held so tenaciously by calcium hydroxide that it cannot be eluted. The following artifice is then available : chromatography is carried out on calcium

hydroxide and the broken-up column is suspended in water, through which carbon dioxide is passed to convert the lime into carbonate. Elution is then easy. (Tóth, unpublished communication.)

Apparatus

A variety of apparatus has been recommended for the practice of chromatography. Of historical interest is that of Tswett (1), shown in Fig. 3, No. 1 : it served for rapid explora-

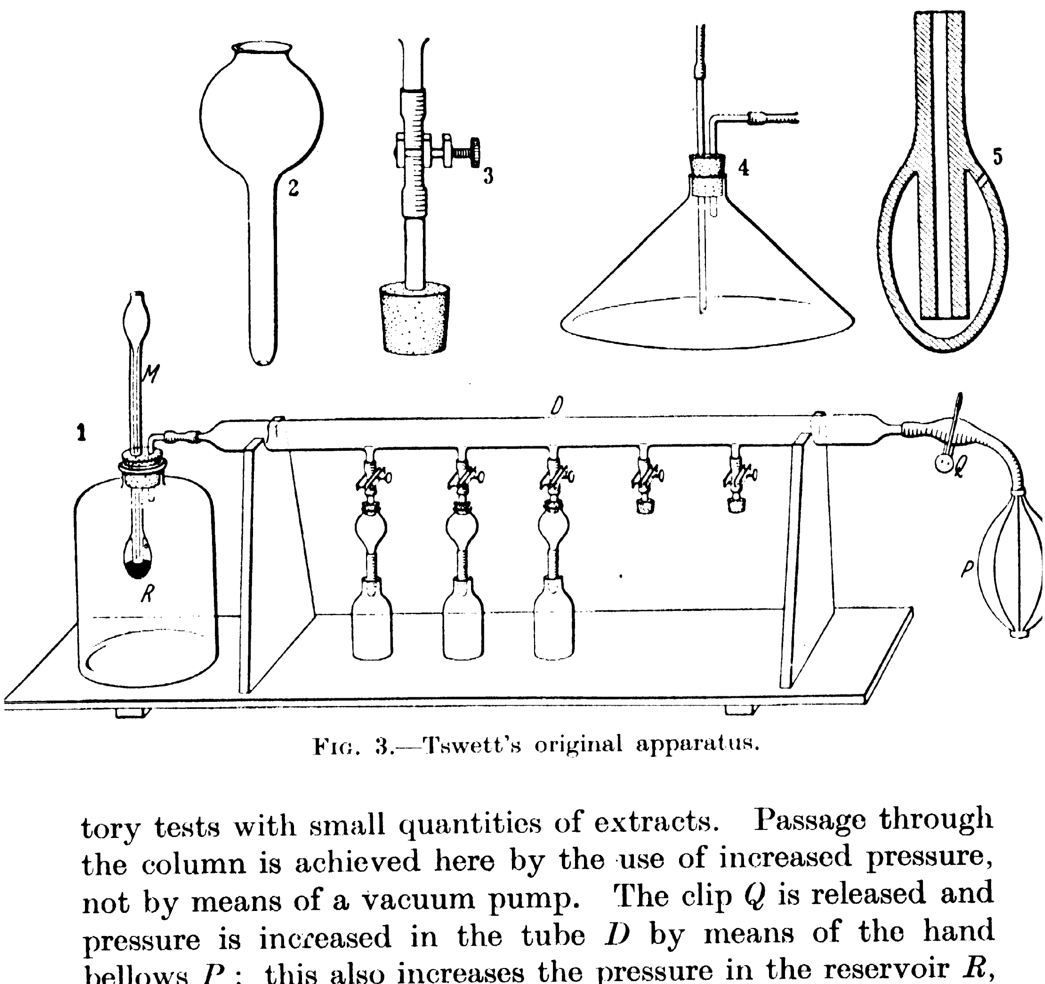


Fig. 3.—Tswett's original apparatus.

tory tests with small quantities of extracts. Passage through the column is achieved here by the use of increased pressure, not by means of a vacuum pump. The clip *Q* is released and pressure is increased in the tube *D* by means of the hand bellows *P* ; this also increases the pressure in the reservoir *R*, as shown on the manometer, *M*. Adsorption tubes of the structure shown in Fig. 3, No. 2, are attached to *D* ; the column of adsorbent is in the lower cylindrical portion, 30 to 40 cm. long and 2 to 3 cm. wide. Other items of the apparatus

are shown in Fig. 3, Nos. 3 and 5. The vessel shown in Fig. 3, No. 4, plays the part of a separating-funnel and is not involved in the preparation of the chromatogram.

To work up larger quantities of material, Tswett (1) used the apparatus shown in Fig. 4. This is identical in principle with what is used to-day; the solution is put in *R*, *A* is the column, of diameter up to 3 cm., and *V* is a filter flask. Extrusion of the column is difficult to manage with a tube of this kind.

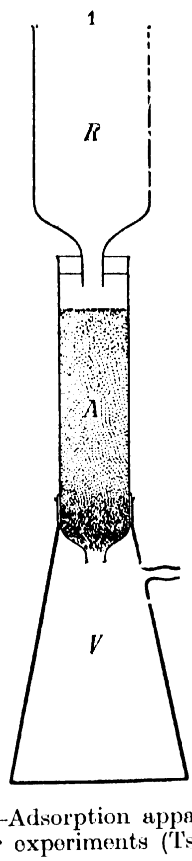


FIG. 4.—Adsorption apparatus for larger experiments (Tswett).

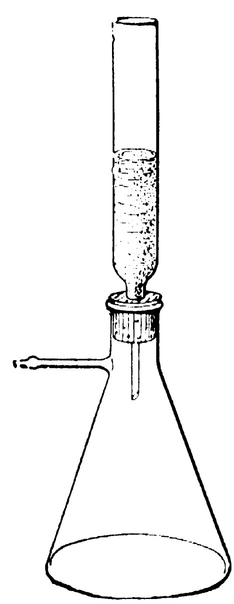


FIG. 5.—Simple adsorption apparatus.

If no specially constructed apparatus is available for testing small samples, then a tube can be drawn out at one end and mounted in a filter flask (Fig. 5). Admittedly the shape of this tube makes it impossible to extrude the column, unless the glass is cut, but this is often not needed in exploratory tests. Furthermore, the individual layers can be washed down gradually and the filter flask changed at appropriate times. (See also p. 76.) An example of this procedure was given some time back by Palmer and Eckles (Fig. 6).

In carrying out the procedure as used at the present day, it is necessary to secure faultless extrusion of an undamaged column. This was first successfully achieved in Dhéré's laboratory by Rogowski (Fig. 7) and also by Vegezzi. In Fig. 8 the actual adsorption tube t (35 by 1·6 cm.) is seen inside the glass cylinder m. The former is fixed by means of a bored cork and can easily be removed. Before the experiment it is closed with a suitable stopper, filled and placed on the perforated plate d (v is glass wool). A modification of this arrangement is due to Winterstein and Stein (1) and is shown in Fig. 9. G is a glass tube, K is fine copper gauze, V is an adapter, D is coarse copper gauze, S is a filter flask (see also Fig. 10).

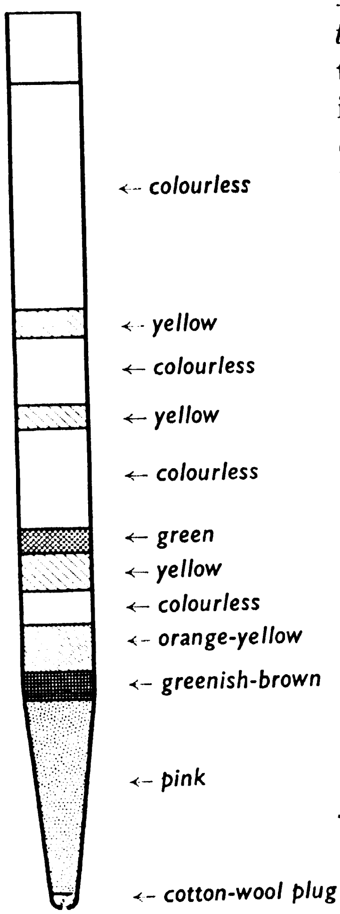


FIG. 6.—Extract of alfalfa, on calcium carbonate in a simple tube, washed sufficiently to cause carotene to pass downwards.

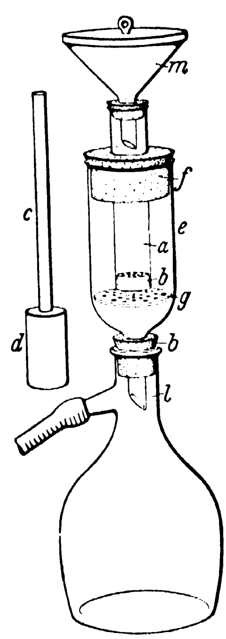


FIG. 7.—Apparatus of Dhéré and Rogowski (illustration from Rogowski). Tube 25 by 2 cm.; height of column, 6 to 8 cm. Above the cork stopper is filter-paper, beneath 1 to 2 cm. of sand. Left, wooden plunger.

If the adsorption analysis has to be carried out in the absence of air, in a stream of nitrogen, for example, the apparatus of Heilbron, Heslop, Morton, Webster, Rea and Drummond is to be recommended (Fig. 11; oxygen-free gas is introduced at *S*) or that of Holmes, Cassidy, Manly and Hartzler (Fig. 12,

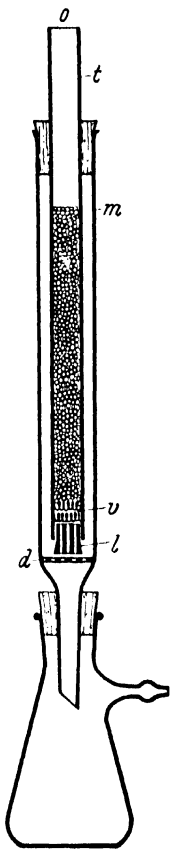


FIG. 8.—Adsorption apparatus of Dhéró and Vegezzi (illustration from Vegezzi).

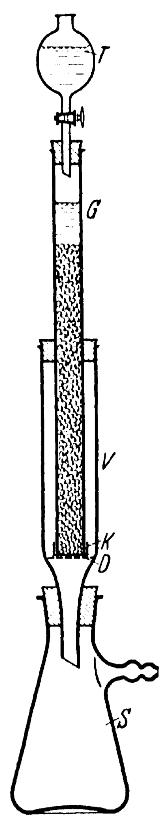


FIG. 9.—Apparatus of Winterstein and Stein.

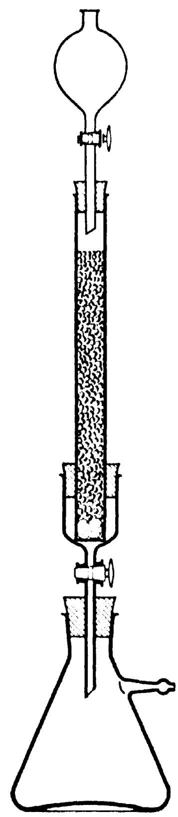


FIG. 10.—Apparatus of Hesse.

p. 60). Koschara (1) used a straight adapter (3 cm. in diameter) with a fused-in perforated glass plate.

Valentin and Franck recommend the apparatus shown in Fig. 13, p. 61, for pharmaceutical investigations; in this the filtrate first flows through a graduated separating funnel and can be released from this at the required rate. For relevant apparatus, see also Euler and Schlenk (2), page 282.

According to our own experience, adsorption tubes with ground-glass joints are particularly useful. One of these was proposed by Kuhn and Brockmann (3) for analytical purposes; the narrow column rests on a plug of cotton-wool (Fig. 14, p. 61; cf. also Fig. 15, p. 61). In the authors' laboratory, the following arrangement has been devised (Figs. 16 and 17,

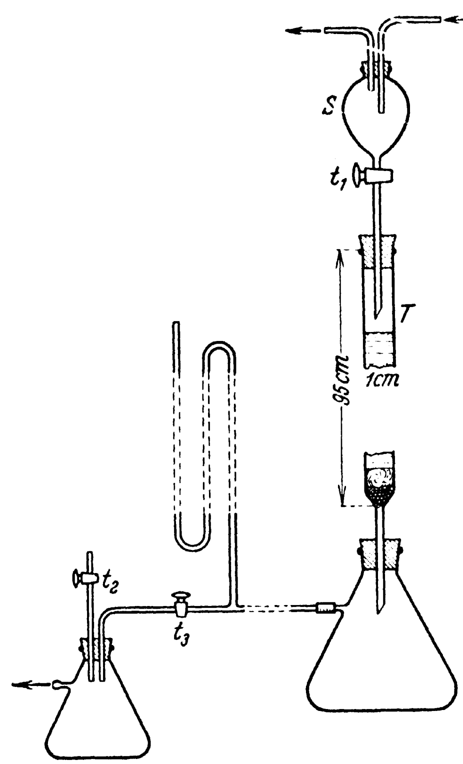


FIG. 11.—Apparatus of Heilbron, Heslop, Morton, Webster, Rea and Drummond for adsorption in the absence of air.

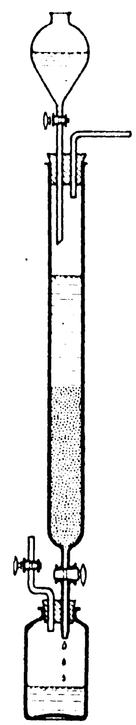


FIG. 12.—Apparatus of Holmes, Cassidy, Manly and Hartzler for adsorption in the absence of air.

p. 62 and facing p. 62). Select a suitable perforated porcelain plate and place on it a plug of cotton-wool, not more than 0·5 cm. high and having a diameter 2 cm. greater than that of the plate. The overlapping portion of the cotton-wool is uniformly folded outwards (over the edge of the plate) and the whole is pushed carefully into the adsorption tube, which is held horizontally by means of the ground-glass joint. The ring of cotton-wool prevents the glass wall being broken when the tube

contents are subsequently extruded. After the adsorption the lower ground-glass connection is removed and the column is pushed out in a horizontal position by means of a wooden pestle (Fig. 30, p. 68). When working with materials that make extrusion difficult—as especially with aqueous liquids—it is possible to dispense with the porcelain plate. The adsorption column may then rest on a layer of cotton-wool which is

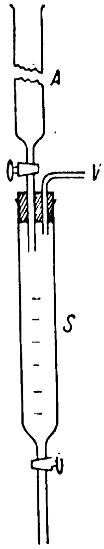


FIG. 13.—Apparatus of Valentin and Franck. A, adsorbent; V, vacuum line; S, separating funnel.

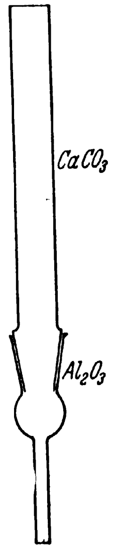


FIG. 14.—Apparatus of Kuhn and Brockmann (3) for analytical work.

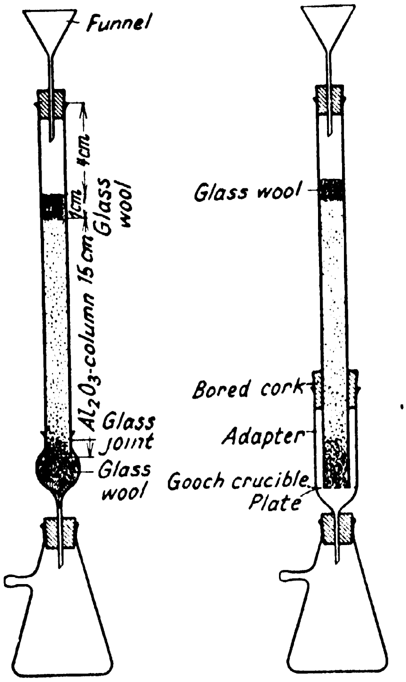


FIG. 15.—Apparatus for the examination of wine (Mohler and Hämmerle).

supported by a few pieces of glass tubing in the lower part of the ground-glass connector, as shown in Fig. 18, p. 62.

Another pattern has also been in use in our laboratory for a long time. It contains a perforated glass plate sealed on to the lower part of the ground-glass connector (Figs. 19, 20, facing pp. 62, 63). The arrangement thus consists of only two parts, which are held together with wire spirals. This involves filling the tube after the ground-glass connection has been made; the lower exit tube must only be disconnected with

the apparatus in a horizontal position. The column is supported on a disc of cotton-wool, about 5 mm. thick, cut off from " block " cotton-wool, with a diameter 3 to 5 mm. greater than that of the glass tube.

For the protection of adsorption tubes that are to be stored, we recommend the arrangement shown in Fig. 21, facing p. 63.

For rapid qualitative investigations of chromatographic behaviour, Schöpf and Becker have recently introduced adsorption sampling tubes (Fig. 22, below). A glass tube of

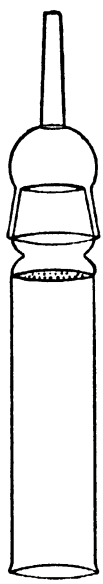


FIG. 16.—Adsorption tube with ground-glass joint and removable perforated disc.

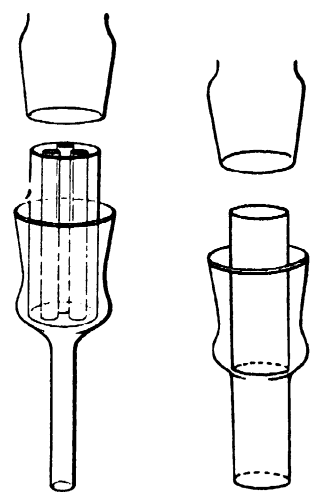


FIG. 18.—Adsorption tubes with ground-glass joint and without perforated disc. (The left-hand model is shown on the more reduced scale.)

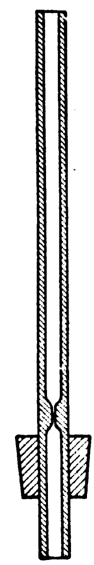


FIG. 22.—Adsorption sampling-tube (Schöpf and Becker).

5 mm. diameter is softened in the flame near one end until it collapses, leaving a capillary 0·5 to 1 mm. wide. A little cotton-wool is pushed in and the tube is filled with adsorbent and submitted to slight suction.

The dimensions of adsorption tubes are generally from 15 to 35 cm. in length and from 1 to 6 cm. in width ; larger sizes have more rarely been recorded. Thus Heilbron, Heslop, Morton, Webster, Rea and Drummond use a tube 95 cm. long, Grassmann one 100 cm. long and 10 cm. wide. Wieland and Probst use one of similar length, but only 1 cm. wide. In the

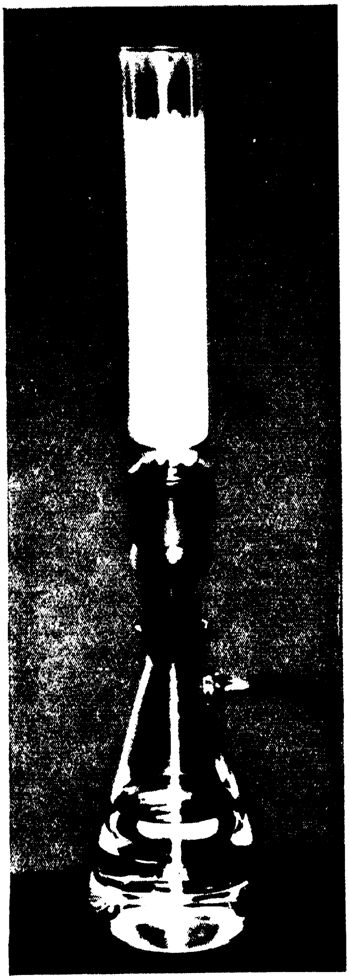


FIG. 17.—Adsorption tube with ground-glass joint and removable perforated disc (*in situ*).

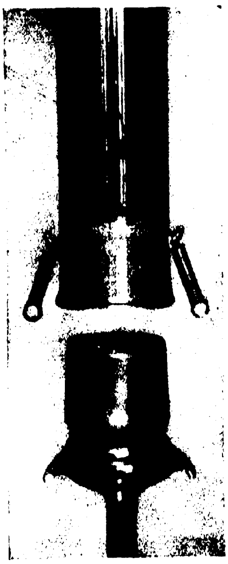


FIG. 19.—Adsorption tube with ground-glass joint and sealed-in perforated disc.

[*See page* 62.]

[*To face page* 62.]

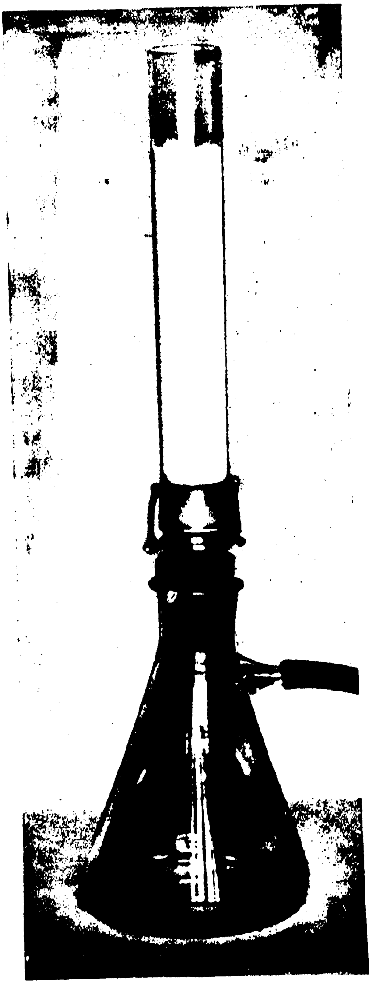


FIG. 20.—Adsorption tube with ground-glass joint and sealed-in perforated disc (*in situ*).

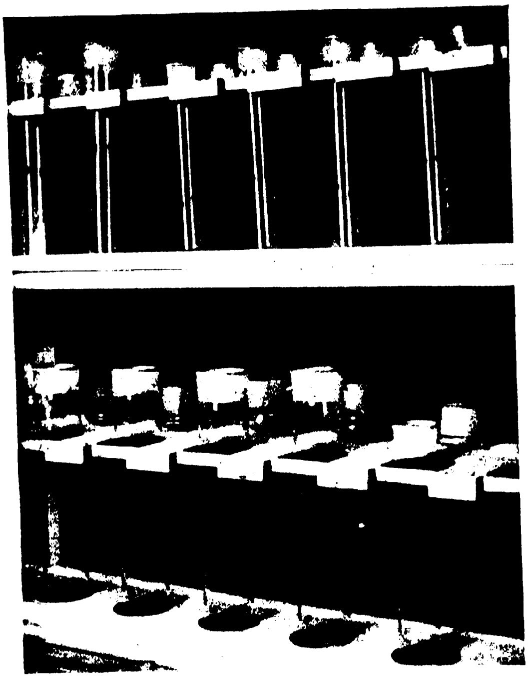


FIG. 21.—Shelves for storage of adsorption tubes.

[*See page* 62.]
[*To face page* 63.]

experiments of Karrer and Strong the column was 80 cm. high. It is clear that a limit is set to increases in the dimensions, in particular by the resistance to the flow of solution and to the extrusion of the adsorbent.

Our models with ground-glass joints have generally the following dimensions (cylindrical portion): 35 by 8, 30 by 6, 25 by 4·5, 23 by 3·5, and 20 by 2 cm.

Selecting the size of the tube is not to be made according to a preconceived plan. Often it would be in the interests of uninterrupted experimental procedure to use a wide column, which would hold as much adsorbent as possible and would retain a large quantity of adsorbed material, without unduly increasing height and consequent resistance. This can indeed be achieved up to a point. Particularly when a simple mixture of substances is involved, or when the formation of separate zones is of no consequence, a short wide tube is to be recommended. Such a shape, however, is unsuitable when the material to be worked up has many components, of which some are only present in small proportions. It is then necessary to turn to a narrow tube, otherwise the zones will be too thin and hardly to be separated from one another.

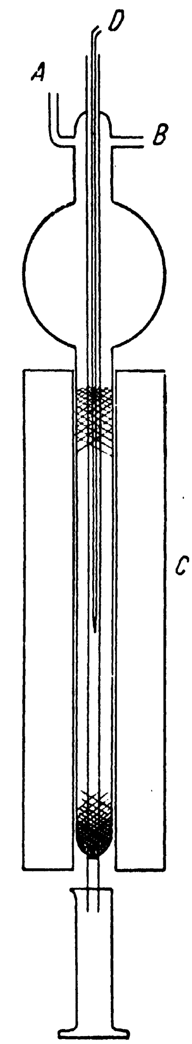


FIG. 23.—Adsorption column, electrically heated. *A*, inlet tube for solution; *B*, nitrogen inlet; *C*, heating jacket; *D*, thermopile (Ralston, Harwood and Pool).

In case of necessity a larger number of columns must be used; corresponding zones are cut out and their eluates combined. On one occasion we have worked up the contents of 380 chromatograms! (Zechmeister and Cholnoky, 3). Or again, a first separation may be carried out in an apparatus of large capacity, and a resulting fraction further split up in a narrow tube for the final analysis.

In making all adsorption tubes care must be taken that the end used for filling is not less in diameter, but rather a shade

greater, than the tube itself; otherwise the column may crumble. Occasionally tubes are recommended that are narrowed towards the base, that is, slightly coned.

In special circumstances the tube may be water-jacketed, for cooling or heating the column (see Boekenoogen 1, for the chromatography of undiluted palm oil, also Kuhn and Ströbele, p. 207). Such an arrangement is, of course, only practicable with a narrow column. Ralston, Harwood and Pool used electrical heating and measured the inside temperature of their silica gel column with a thermopile (Fig. 23, p. 63). Cerecedo and Hennessy used a metal tower, 150 by 28 cm., and raised the contents to 75° C. with hot water (p. 251).

Large-scale Apparatus

An arrangement recommended by Winterstein and Schön (3) exceeds the dimensions of the chromatographic apparatus normally used in the laboratory. It holds several kilograms of sucrose and contains a thick "Porolith" filter plate (Filterwerk, Meissen) with fine pores. A chromatogram of chlorophyll on sucrose is finished in 1½ hours in this apparatus, and the process is even quicker with alumina. A glass-cylinder, *G*, 50 cm. high (Fig. 24), diameter 12·5 cm., is fitted at the bottom with a ground-glass flange; it is fixed down by means of the screws, *S*, and the ring, *R*, on to a "Porolith" filter-plate provided on the outer edge with an india-rubber ring. This secures uniform suction. The filter-plate rests on the metal housing, *M*, which is supplied with an outlet tube, *A*. This is mounted in a filter-flask by means of a rubber disc. The apparatus, which holds about 6 kg. of adsorbent, stands on a tripod.

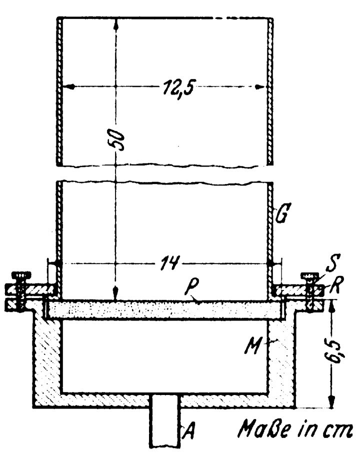


FIG. 24.—Apparatus of Winterstein and Schön (larger model). (Dimensions in cm.)

The second apparatus of Winterstein and Schön (3) is designed for use with smaller quantities of material (Fig. 25).

The tube (25 by 7·5 cm.) has a ground flange at the bottom and this sits on a porolith plate, *P* (9·5 cm. diameter), provided on the outer edge with an india-rubber ring. The plate rests on a glass funnel fitted with a suitable ground edge. The three parts of the apparatus are kept together by means of two metal rings, *R*, and the screws, *S*.

To fill the apparatus the adsorbent is stirred with petroleum, for example, and sucked dry in small charges; mechanical separation of the chromatogram layers is achieved by removing portions with a small shovel.

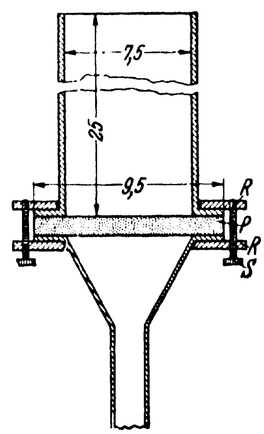


FIG. 25.—Apparatus of Winterstein and Schön (smaller model).

(Dimensions in cm.)

Manipulation of large quantities is generally easy to carry out in a narrow percolator (one, for example, 85 cm. high, 12 cm. wide at the top and 7 cm. wide at the bottom) closed at the bottom with cotton-wool, gauze and filter-paper, and fitted into a filter-flask connected to one or two good water-pumps. We use rather wider percolators, of a slightly conical build, especially for the first enrichment of the adsorbate from large volumes of liquid; the column is covered with filter-paper and rests on a porcelain plate. Removal of the layers with a spatula is generally carried out with the column horizontal. If the percolator is not narrowed at the top, it can be turned upside down and the cake-like mass knocked out by hitting the opening with the palm of the hand.

As far as we are aware, nothing is yet known about arrangements in use on a manufacturing scale.

Apparatus for Micro-Chromatography

In order to reduce very considerably the amount of substance to be treated (down to 0·5 to 1 μg.) it is particularly the diameter of the tube that must be chosen sufficiently small. Tswett (1) himself went down to as low as 2 mm. Hesse (1) used a micro-tube (Fig. 26, p. 66) and the dimensions of his

column were 30 by 2 mm. Schwab and Jockers (2) used similar tubes, internal diameter 4 to 7 mm., with widened tops.

Still narrower than Hesse's adsorption columns are those of Becker and Schöpf (Fig. 27, below). A thick-walled capillary (internal diameter 1 mm.) is expanded at the bottom to take a small plug of cotton-wool; for ease of filling the solution, the capillary is fused to a glass tube of 4 to 5 mm. width. The tube has a ground-glass plate as foot; it is placed in a straight adapter on a perforated plate and secured in a suction flask. After the experiment the tube itself is cut up.

Fig. 26. — Micro-adsorption tube (Hesse).

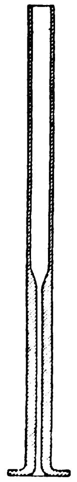


Fig. 27. — Micro-adsorption tube (Becker and Schöpf.)

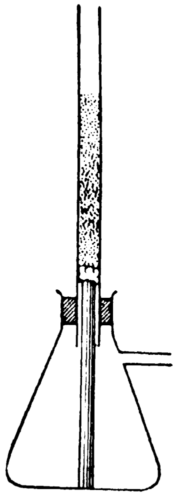


Fig. 28. — Arrangement for micro-chromatography (Willstaedt and With.)

Recently Willstaedt and With (1) have also concerned themselves with micro-chromatographic experiments; they have proposed the apparatus shown in Fig. 28.

The column contents rest on a cotton-wool plug in a tube of 5 to 10 mm. diameter, supported by a glass rod, which stands on the bottom of the filter-flask and can be used at the end of the experiment to press out the column. To prepare the column, a layer of dry alumina, 5 mm. high, is introduced, moderate suction being applied: the pump is then connected and the tube is filled with petroleum. As the liquid is slowly passing through, alumina is introduced through a dry

funnel and allowed to sink down the column of petroleum. Solvent is added by a pipette at such a rate that the column is always under liquid. Finally, the column contents are covered with two filter-papers, especially with the larger tubes. With narrower tubes, having diameters between 1 and 5 mm., the adsorbent is introduced by means of a large Pasteur pipette. The glass rod need not be used in such instances.

Examples of micro-chromatographic procedures are given on pages 157, 172. The main problem of micro-chromatography lies not so much in the assembly of the apparatus as in ready identification of the adsorbed substance.

Experimental Procedure

Formation of Adsorption Column

An important condition for successful chromatographic analysis is a uniformly and well packed tube. For if the powder is unevenly distributed, cracks and channels appear, the solution finds its way through these, and the large number of essential individual adsorptions and elutions do not take place. These crude faults are easily avoided. Rather more practice is necessary to secure such homogeneity that the individual zones of the chromatogram are not curved or twisted. Irregularities like those shown in Figs. 55 and 56 (p. 318) are permissible, whereas Fig. 57 illustrates a badly filled tube.

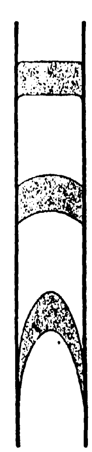


Fig. 29.—Schematic representation of curved layers.

Ideal conditions of zone-formation are especially rare in work on an intermediate scale. They are, however, not really essential in such instances ; it is sufficient if the position of the individual, clearly demarcated regions facilitates the subsequent cutting of the column. A common occurrence is for the liquid to pass down along the cylindrical glass surface at a greater speed than down the middle of the solid phase. Then there appears an arrangement like that represented in Fig. 29 ; thin layers lying near the bottom of a wide adsorption tube assume a cap-like shape.

In order to remove the coarser contaminants from a powder, we recommend that it be sifted, preferably just before the tube is filled. Next the apparatus is furnished with the necessary support for the column—cotton-wool plug, glass-wool, porcelain plate, sintered glass-plate, etc. (p. 60), and the adsorbent is shaken portion by portion into the tube, which is held in a vertical position.

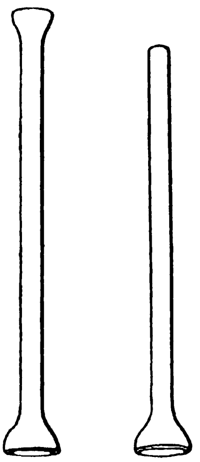


FIG. 30.—Wooden pestles.

In tubes with a diameter up to 1 cm., each individual portion of adsorbent, of which the first should be larger than the remainder, is pressed down by means of a flattened glass rod. In larger apparatus, such as are mostly used for preparative work, a smoothly turned cylindrical wooden pestle is used ; the area of its working end should be two-thirds to three-quarters that of the tube. It is convenient to construct the appliance (of walnut wood) for use in tubes of two different sizes (Fig. 30) ; in any event, the smaller end can be used for extrusion of the column at the end of the experiment. If the apparatus shown in Fig. 20 (facing p. 63) is used, then the end of the pestle must be only very little less in breadth than the width of the tube. The end should be circular, sharply coned and slightly convex in the middle.

The pressing down of the adsorbent should be carried out by short vigorous tapping, not too violent, from a height of 3 to 6 cm. As the pestle is raised, a slight vacuum is created and this causes certain adsorbents to be sucked back with formation of a cloud of fine powder. To prevent this mishap Rogowski some time ago recommended that the wood be tubular ; it is, however, sufficient to give a slight rotary movement to the pestle as it is raised. Uniform filling of the tube is also assisted if the tube, held vertically, is slightly turned with the left hand each time the pestle is raised.

Example : 550 g. of calcium carbonate were introduced into a tube 30 by 5·5 cm., to a height of 24 cm. ; it was divided into one portion of 50 g. and 20 portions of 25 g. At each addition the column was tapped 15 to 20 times and the whole operation took from 10 to 15 minutes.

From one-third to one-fifth of the tube volume is left free for the solution. The amount of adsorbent is usually generous compared with the amount of substance to be taken up; departures from this principle are only made in special circumstances. Thus Strain (1) used for partial separation of α- and β-carotene an amount of magnesia not quite sufficient to hold all the pigment. The less easily adsorbed α-isomer was thereby concentrated in the filtrate.

After the powder has been introduced into the tube to the desired height, this is fitted into a filter flask, to which a pump is attached and allowed to run, while the column is again pressed down with the wooden pestle; the circumference of the upper surface, especially where it is in contact with the glass surface, is gently and uniformly tapped—not pressed. This last step takes about a minute; it should be continued just so long as withdrawal of the pestle produces a little cloud of dust, but no longer. In this way air bubbles and channels are avoided when next the solution is poured on. If, however, too hard a surface is produced, as with calcium carbonate and similar materials, the column may then crack, and cause large lumps of adsorbent to float in the liquid.

Pressing down the adsorbent is often particularly tricky in the more capacious tubes, though we ourselves fill even large percolators by this method. Winterstein and Stein (1) proceed differently; the apparatus, kept vertical, is connected to the pump and a suspension of the adsorbent in petroleum is introduced in small portions. After each separate addition suction is applied, first gently, then more strongly: at all times the surface of the column must be covered with petroleum. The method of filling with 3 kg. of sucrose the percolator described on page 64 was very wearisome and took 2 to 3 hours; the pressure procedure was quicker.

Castle, Gillam, Heilbron and Thompson state that a calcium hydroxide column suitable for analysis of vitamin A concentrates must be prepared in the following way. The material is vigorously shaken with a few volumes of petroleum and the suspension is then allowed to stand for half an hour, with occasional gentle rotation to expel air bubbles. The tube is filled portion by portion and each portion is added only when the previous one has completely settled. Preparation of such

a column takes several hours. Holmes, Cassidy, Manly and Hartzler remove gas bubbles by tapping, Schwab and Jockers (2) warm an aqueous suspension of alumina to 70° to 80° before filling. See also Euler and Schlenk (2) (p. 282).

According to our experience, filling by means of a suspension does indeed in some instances, though not in all, lead to a more uniform production of pigment zones ; on the other hand, especially with calcium carbonate or lime, there is the counterbalancing disadvantage that longitudinal channels may form in the column when it is extruded.

In suitable circumstances, especially when filling has to be expedited, we have introduced into the tube dry calcium carbonate or hydroxide, portion by portion, under continuous suction, pressing it down rapidly. In this way a good horizontal stratification is secured, but the column may not be extruded later, as it tends to fall into a series of pieces curved outwards away from the mouth of the tube. Removing the tube contents with a spatula or preparing a " liquid chromatogram " is of course permissible.

If separate layers of two adsorbents are filled into a common tube, the lower layer is pressed down in the usual way ; the pestle is then wrapped in a piece of clean linen and the tapping is continued for a little time, with the pump running. The walls of the glass tube are then carefully freed from small particles and the second adsorbent is introduced. If this precaution is omitted, the chromatogram may under some conditions be markedly affected at the surface where the two adsorbents are in contact.

Introduction of the Solution

When the tube has been prepared, as described, the pump is allowed to continue running and the first portion of the liquid is poured in without delay, in such a way that the whole surface is moistened and covered as nearly simultaneously as possible, yet without stirring it up. This can be managed without any special manipulation, but it is permissible to use a tap-funnel or any device for automatically regulating the flow. Examples of these are given on pages 58 to 62, as

also of operations in the absence of air. A long-necked flask, filled with the solution, of which the neck is immersed in the liquid, performs a similar service in lengthy experiments ; even more convenient is Körner's arrangement (Fig. 31), which is suspended over the apparatus in a metal ring.

It is most important that the upper surface of the column remain permanently covered with liquid, from the introduction of the first portion to the end of development. If this is not seen to, in some circumstances the column dries so quickly as to shrink. If fresh liquid is then poured on, it no longer passes uniformly through the whole section, and part of it hurries ahead down the walls of the glass tube. For this reason the liquid phase must only disappear from the part of the tube above the column when extrusion is just about to be undertaken.

If an evenly pressed-down column while still dry is suddenly brought in contact with a liquid, two mishaps may occur with certain materials : first, small particles are spattered up from the upper surface and, secondly, small cracks may appear, thereby disturbing the solid structure.

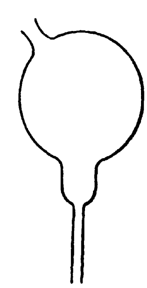


FIG. 31.—Körner's arrangement for automatic filling.

The column is protected by laying on it a circle of filter-paper ; just before the experiment this is moistened with the solvent. Tswett placed on top of the tube contents a plug of cotton-wool saturated with the pure solvent, leaving it there for a while and only removing it immediately before pouring in the solution. In this way there is a gradual permeation of the sensitive upper surface with the vapour of an organic solvent. Sometimes it is advantageous first to moisten the whole column with the solvent, suction being applied. In numerous other instances all the processes mentioned may be omitted, especially when a narrow tube is being used. Moistening of the column has been found undesirable in the chromatography of cellulose acetate.

Finally it may be remarked that certain authors have recently advocated an improved form of the method originally used by Tswett, in which pressure is applied, instead of suction. Accounts of this are given by Sannié (Fig. 32) and by Potts and

Koch ; Mark and Saito used compressed air (see also p. 197 for cellulose acetate ; cf. also Fig. 23, p. 63).

To use Sannié's apparatus (Fig. 32), prepare a moist column and then cover this with a little solvent. Close R_2 and R_3, open R_1 and admit solution into A. Then close R_1, open R_3 and regulate the flow of liquid from A into T so that about as much enters as runs out from the end of the column. Introduce the developer in the same way. Pressure is applied at M, by way of a bottle carrying an open tube, 80 cm. long and filled with mercury.

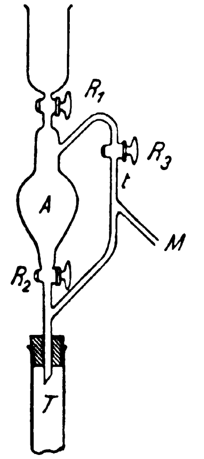


FIG. 32.—Sannié's arrangement for working under pressure.

Development of the Chromatogram

Development is carried out with copious quantities of a pure solvent, which may be the same as the main solvent or different. The second course is chosen when an increase in eluent activity is required, that is to say, when the zones are separated too slowly, or not at all, by the solvent used for the original solution. Development is continued until the appearance of the column is optimal and all the zones, or those with which one is especially concerned, look as if they could be cleanly cut out. In the course of development the surface of the adsorbent must still be continuously covered with liquid ; damage will be done if this is omitted even for quite a brief interval. Figs. 54 to 56 (p. 318) illustrate the progress of a development.

Kuhn and Weygand purified a crude solid preparation of flavin by grinding with alumina and pressing down on to a previously prepared column of alumina. Solution and development took place as part of a single operation, namely, pouring in a mixture of wood spirit and xylene.

The description of a developed column can conveniently be given according to the scheme used in the Special Section ; in this the figures on the left give the thicknesses of the zones in millimetres (example on p. 130).

Assessment of a Chromatogram. Effect of Impurities

In assessing a chromatogram, the following points must be taken into account. The appearance is to a considerable extent independent of the impurities present, and indeed the manifold uses of Tswett's method depend largely on this fact. But there are also innumerable instances when an alteration is observed in the normal appearance of the column; the zones appear unusually wide and pale, as if an eluent were in use. It is indeed the fact that high concentrations of certain impurities may markedly depress the surface affinity of the solid phase for the solute. The adsorption isotherm then differs markedly from that of the pure compound and in such a manner that elution processes take place during the experiment. This is observed, for example, in the analysis of animal lipochromes if they are accompanied by large amounts of fat, cholesterol and so on. More rarely an impurity may completely prevent from being fixed on the column substances that in the ordinary way would be held there (cf. Figs. 63 and 64, p. 321).

Repetition of the adsorption from one to four times generally gives the desired result, for the absolute quantity of the impurity sinks rapidly from one column to the next. If absolutely necessary, the crude extract can first be submitted to some other kind of preliminary purification; this will be of advantage particularly when the ratio of the desired compound to the total solids is very unfavourable. On some occasions a heavily contaminated substance is only ready for chromatography after some preliminary manipulation.

Especially pure preparations generally show strong adsorptive properties, and it may even happen that the high degree of purity may render elution difficult or prevent it altogether.

The great number of possible combinations of factors include the alternative circumstance that the conditions just described may be completely reversed; the presence of contaminants may favour the fixing of a substance on the column. The column is, so to speak, mordanted, and adsorption is promoted. In such an instance it is not the material forming the column that is to be regarded as the adsorbent, but rather the combined system column-contents + contaminant.

The fixing of a third substance is termed by Schöpf and Becker "secondary adsorption" (cf. also Koschara, 4, 5).

Schöpf and Becker have shown that erythropterin (p. 170) is adsorbed very strongly by alumina from a crude solution in aqueous pyridine, whereas this fails with the pure substance. The amount of the active, nearly colourless contaminant can only be slight, for it is insufficient to fix the whole of the pigment ; a portion thereof runs through the column and can thereby give a misleading impression of heterogeneity. The fixed portion of the pigment is slowly eluted with dilute aqueous pyridine and is not readsorbed if passed through a fresh column of alumina. The contaminant remains in the same region of the first column as was formerly occupied by the erythropterin ; it is revealed by a slight brown coloration and a violet-blue fluorescence under the quartz-mercury lamp. Further study of artificially induced secondary adsorption should lead to interesting results.

Finally it must be pointed out that adsorptions from aqueous solutions can be influenced by the presence of salts, so that variations in salt concentration may lead to graded variations in the extent of adsorption or elution (cf., for example, Koschara 4, Ruggli and Jensen 1, p. 193).

Extrusion and Division of the Column. Elution

The column contents must be neither too moist nor too dry if the adsorbent is to be extruded easily and without any crumbling. After development is finished, the pump is allowed to go on running until there is no more liquid above the column. Suction is still applied for a short while, nitrogen being led in if very oxy-labile substances are being handled. The right degree of moistness is often reached by closing the top of the tube with the palm of the hand for 15 to 20 seconds, leaving the pump still running.

Extrusion is best carried out with the aid of the wooden pestle illustrated on page 68. The tube is held horizontally immediately over plain or glazed paper. The operator is seated with one end of the pestle pressed against his body ; the larger-sized tubes must be held in both hands. If extrusion is difficult or impossible, the adsorbent is removed with a spatula

or spoon, a procedure that is nearly always necessary in large-scale experiments (p. 65). To loosen smaller columns, it is sufficient to remove the tube from the apparatus, invert it and then let it fall several times from a height of 1 or 2 cm. on to an ordinary plate covered with a cloth. The column moves slowly towards the opening of the tube and can then be easily pushed out. Furthermore, individual portions of the column can be initially demarked with circular pieces of linen, so that it is possible to extrude any required part of the column separately.

A scalpel is used for cutting the column, which must be carefully carried out; the column must *not* be cut systematically at right angle to its long axis. First unloaded portions of the column are removed; then the separated large portions are taken in the left hand, and scraped at an acute angle to the axis until the white and the only slightly coloured portions have been carefully removed. This is necessary because it permits separation of curved or irregular zones. With complicated columns one indulges almost in a form of sculpture!

The individual homogeneous portions of the column are coarsely broken up with the scalpel and then immediately dropped into the eluent, which is standing ready, and stirred round. Solution generally takes place very quickly: it is only rarely necessary to raise the temperature. The suspension is filtered, preferably on a sintered glass funnel; and the filter-cake is washed. Control of homogeneity is best carried out by repeating the chromatography.

No generally valid rules can be given for further examination; any physical, chemical or physiological method is available to suit the nature of the substances involved; the same applies to their isolation.

Ruggli and Jensen (1) used an original procedure in the chromatography of certain water-soluble coal-tar dyes. Each partial eluate was immediately used to dye some mercerised cotton-wool and then re-extracted with boiling water for final isolation in the solid form. Thus the selective action of the adsorption column was supplemented by the differential fixing of the pigments on the fibres.

Liquid Chromatogram

Besides the previously described course of an adsorption experiment, another method of working is very frequently followed ; this has already been indicated on page 54 and is characterised by the fact that the column is neither removed from the glass tube nor cut up. The procedure is rather to drive the whole of the adsorbate—or portions thereof—through the column and to collect the separate fractions in the filtrate by appropriate changes of the receiver. The series of filtrates constitute the " liquid chromatogram " ; this is an equally convenient mode of procedure.

The principle is applied in practice in a number of ways, according to the adsorption forces at work. For example, a normal chromatogram may first be prepared and then developed so energetically that the components pass into the filtrate one after the other. This may either be done with a single eluent or with a succession of solvents having increasingly powerful eluent actions ; each removes particular pigments from the column. If one had available an eluent strongly selective for each individual layer, the conditions would be ideal for this kind of experiment.

With certain grossly contaminated crude solutions the preparation of a liquid chromatogram is sometimes unavoidable, because a stable chromatographic picture may be impossible of achievement. In such circumstances there is continuous passage of solutes from the beginning to the end of the analysis. There is no true development ; portions of the filtrate are simply collected separately. Similar conditions may be intentionally allowed to arise if, for example, an adsorbent has been chosen that is too weak to retain all the contents of the solution.

The procedure just described does not, of course, permit the separation of thin, closely packed zones, especially when they contain substances of similar nature : substances that have been separated appear together again in the filtrate. This can also occur with well-formed chromatograms, if development is carried out too violently, spoiling the separation that has already been achieved. For the substances held in the top portion of the column pass rapidly into solution, are washed

down and mixed with the substances in the lower portion before these have been able to leave the column. If this happens with colourless substances, whose separation cannot be directly observed, there may be an illusory appearance of non-separability, especially if certain zones of the chromatograph are strongly curved.

In spite of this limitation, the liquid chromatogram is an indispensable aid in working up many mixtures. The filtrates so obtained can also be submitted to " normal " chromatography.

Effect of Hydrogen Ion Concentration. Adsorption and Elution in Aqueous Solutions

It is known, especially from the classical work on enzymes carried out by Willstätter and his school, that adsorption of an organic substance from an aqueous solution on to a solid phase of inorganic constituents is influenced and determined by hydrogen ion concentration. Relatively small shifts in *p*H can produce powerful effects and may, for example, break up the adsorbate. Similar phenomena occur on the surface of each individual granule and are largely independent of the outer form of the total adsorbent mass. They also play a part in the Tswett experimental technique.

It follows that in certain circumstances the work must be carried out in a buffer-solution. Koschara (4, 5) was the first systematically to investigate and to lay down the specific conditions for particular circumstances. Reference may be especially made to his collective investigations into bleaching earths.

With aqueous solutions the use of bleaching earths allows one to conduct operations over a wide range, namely from 5-*N* mineral acids to a *p*H of 11. Stronger alkali precipitates hydroxides, which block the filter. Elution is generally carried out from the intact column simply by raising or lowering the hydrogen ion concentration. Thus, for example, adsorption is carried out at *p*H 7·6, washing at 8·3 and development at 9·2. It has happened that the succession of two zones has been reversed by a shift in *p*H of 0·3. These relationships often establish good conditions for the preparation of a " liquid chromatogram " (p. 76).

Sörensen's phosphate and borate buffers have been used; ammonium chloride and ammonium hydroxide buffers are less successful. Aqueous acetates of the alkali metals are to be avoided with bleaching earths; with alumina the buffer solution can usefully be diluted with wood spirit.

If the alkalinity of the buffer solution is not sufficient to compass elution, a 2 per cent or stronger aqueous solution of pyridine can be used, or an even more active mixture of pyridine and acids. Elution is, naturally, determined not only by the medium, but also by the chemical nature of the adsorbent; thus a pigment which was eluted at *p*H 8·3 from bleaching earth XXF could only be removed from a frankonite KL column at *p*H 9·2.

The use of bleaching earths should first be tried out in neutral or acid solution. "Neutral adsorption" can, indeed, only be achieved if the adsorbent itself has a true neutral reaction, that is, if it has not been manufactured with the use of acids. The association in the column of neutral earth with acid solution is unsatisfactory; evolution of carbon dioxide may give rise to fissures. On frankonite, however, acid solutions can be employed, though it generally suffices to use a neutral solution with the acid earth.

A column of frankonite KL and its adsorbate must be plentifully washed with the buffer solution chosen; it is only then in proper condition for being smoothly developed. Although such washing does not generally elute the pigment or cause it to move downwards, yet the column is altered to the extent that local "acid adsorbing" regions disappear. If on the other hand the column has been insufficiently rinsed with the buffer solution, the eluent subsequently used will, it is true, dissolve the pigment and carry it down the column, but the zone will then suddenly come to a halt and will thereby indicate the position down to which the preliminary washing was effective and below which "acid adsorption" is still able to proceed.

A cylindrical suction-filter is a convenient apparatus to use, the water being kept at an adequate level. Suction is first carried out gently (30–50 cm. water pressure); later the pump is allowed to run vigorously. A pertinent description of the procedure is given under the isolation of lyochromes (p. 168)

and uropterin (p. 175) from urine. For chromatography in aqueous solution, without the use of buffers, see anthocyanins, page 177.

Special Methods for Chromatography of Colourless Substances

There is no necessary connection between colour and the arrangement of substances according to differences in adsorption affinity. Rather is the fractional adsorption of pigments simply a special instance of a general phenomenon, experimentally the most convenient because the course of the experiment can be followed with the eye. Tswett (1) himself had already written of the " invisible chromatogram." Recently Koschara (4) recommended that the historical term " chromatography " should not be used in connection with colourless materials, and should be replaced by " adsorption analysis." We cannot accept this proposal, for the essential feature of Tswett's idea—passage through the adsorbent only in one direction—is valid for experiments with colourless substances.

At present the following methods are available for chromatography of colourless or slightly coloured materials :

1. An empirical procedure.
2. Marking a colourless zone with an indicator.
3. Chromatography following chemical conversion of a colourless mixture into coloured substances.
4. Making the chromatogram visible by means of ultraviolet light.
5. Making the chromatogram visible by means of a colour reaction.

One is generally anxious to make possible some immediate observation, by inducing the appearance of some colour or other optical phenomenon. If this succeeds, any of the procedures available for pigments can then be used for dividing the column.

1. Empirical Procedure

This is practised by simply cutting the column into several sections, although no visible indication is toward, and then examining the several eluates. After such a step has thrown some light on the fractionation, a planned experiment can then

be similarly carried out with the same material. This obviously applies also to filtrates, several portions of which can be collected and separately tested. In many instances liquid chromatograms are prepared in an empirical manner.

2. Marking a Colourless Zone with an Indicator

Tswett (1) himself recommended that the colourless solution should be treated before analysis with a pigment, whose place on the column is precisely known relative to that of a colourless constituent. The position of an invisible zone is thus indicated by the pigment, as is also its passage downwards during development and completion of the chromatogram. This procedure is quite practicable, but is hardly universally applicable (cf. also Winterstein and Stein 1). In any event the adsorption phenomena must be very exactly studied and the procedure carefully laid down.

In some recent investigations of Brockmann (3) and of Brockmann and Busse there are pretty examples of this technique. To isolate vitamin D_3 from fish-liver oils (for details, see p. 260), many pigments were examined for their suitability as indicators on the column, in relation to the adsorption isotherms and the chromatographic behaviour actually of vitamin D_2. Due consideration was also given to the influence of the physiologically inactive contaminants occurring in liver oils. "Indicator Red 33" was found to work best, for the red zones of the chromatogram were the highest in vitamin content. When the pigment has become concentrated in a narrow zone development is stopped and the column is cut.

In favourable circumstances the indicator is provided by Nature herself; thus toad venom is accompanied by a pigment that is adsorbed in precisely the same part of the column as the main toxin, bufotalin (Wieland, Hesse and Hüttel, *v.* p. 230; cf. also Tschesche and Offe, p. 233, and Duschinsky and Lederer, p. 272).

3. Chromatography after Chemical Transformation of a Colourless Mixture into Coloured Substances

The principle of this device consists in attaching to the substance an easily removable chromophoric group, before

carrying out the adsorption analysis: this transforms the operation into a separation of pigments. The procedure is due to Strain (4) and was first used on certain carbonyl compounds. These were condensed with 2 : 4-dinitro-phenylhydrazine: subdivision of the mixture of hydrazones was conducted on talc, fibrous alumina, alumina, aluminium phosphate, tertiary magnesium phosphate or fuller's earth. (Magnesia and other alkaline adsorbents bring about decomposition of the adsorbate.) This useful procedure involves a relatively considerable expenditure of time.

Examples: (*a*) A solution of the dinitro-phenylhydrazones of β-ionone and camphor in light petroleum is worked up on talc; the ionone derivative remains at the top, the camphor derivative descends to the bottom of the column.

(*b*) Separation of the dinitro-phenylhydrazones of geronic acid and levulinic acid in petroleum on talc. Above, the hydrazone of the levulinic acid, $CH_3—CO—(CH_2)_2—COOH$, below that of geronic acid, $COOH—C(CH_3)_2—(CH_2)_3—CO—CH_3$.

The dinitro-phenylhydrazones are eluted with spirit and can be split up in aqueous, dilute acid or glacial acetic acid solution by means of a dicarbonyl compound, such as glyoxal, methylglyoxal or diacetyl.

(*c*) The method has also been applied to the picrates of tetra-alkyl ammonium hydroxides.

A process similar in principle has been followed by Lederer in separating phenols (private communication): the aqueous solution is treated with ferric chloride and the deeply coloured solution is chromatographed on alumina. Phenol, resorcinol, catechol and phloroglucinol can be differentiated in this way.

4. Ultra-violet Light as a means of making a Chromatogram Visible

This procedure is particularly fertile and capable of development; it depends on the fact that numerous colourless substances exhibit a vigorous fluorescence in the light of the quartz mercury lamp. The phenomenon was applied to the Tswett technique contemporaneously by Winterstein and Schön (1) and by Karrer and Schöpp (2). The last-mentioned introduced the term "Ultra-chromatography"; Grassmann and Lang speak of a "fluorescence-chromatogram."

This method permits localisation on the column of fluorescent substances and their separation from materials that are not excited by ultra-violet radiation; in favourable circumstances it is also possible to observe individual regions of different tints and different degrees of luminosity. A blue fluorescence is generally non-specific.

A further advantage lies in the fact that formation of such zones may also occur on adsorbents that are dark in colour or appear dark when moist. Correct division of the column can in such instances be cleanly carried out. A pretty phenomenon is the passage of fluorescent substances into the filtrate: conditions for the observation of a liquid chromatogram are thereby achieved.

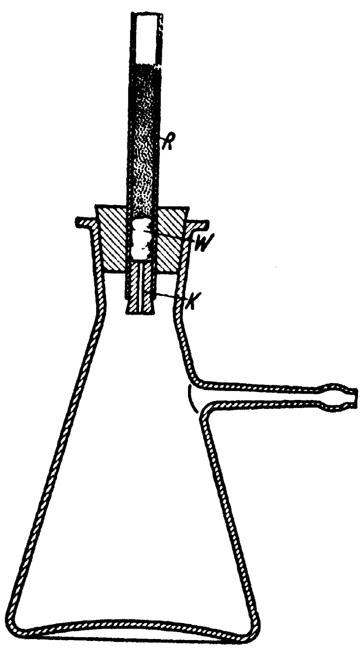


FIG. 33.—Adsorption tube of quartz (*in situ*). (R = tube 16 cm. long; W = cotton-wool; K = cork. After Grassmann.)

The conditions of the experiment are theoretically favourable if a quartz tube is used in, for example, the simple arrangement shown in Fig. 33.

Karrer and Schöpp recommend that experiments on a small scale should be carried out with a tube having plane parallel walls and a quadrangular cross-section. Use of a quartz tube is always expensive, and also makes the use of ground joints very difficult; it is, moreover, in our experience not necessary, if the fluorescence is at all marked. Glass is almost always satisfactory, especially soft soda glass. Further, the column can be illuminated by ultra-violet radiation after extrusion; this avoids the need for any special apparatus.

Irradiation is carried out in the dark-room and any good ultra-violet lamp (analytical quartz-mercury lamp) is suitable as the source of illumination. In our laboratory the new Hanovia type of portable mercury lamp has behaved very well (see Fig. 34). The cover is made of dark Wood's glass which only transmits radiations in the neighbourhood of

FIG. 34.—Division of chromatogram under the portable quartz-mercury lamp, carried out in the dark-room.

[*To face page* 82.]

366 mμ : this avoids the need for using a special screen. The full capacity of the roomy half-sphere (diameter 36 cm.) can be used. The column is extruded on to black paper, and cut under the shade ; the operator looks through a " sight-and-light " and need not wear protective glasses.

Even curved chromatograms can be worked up quite well with the aid of a scalpel. The " empty " portions of the column often appear dark brown or black, the adsorbate being illuminated and in good contrast. However, an empty column may also show fluorescence, according to its chemical properties.

It must be noted that the colour of the fluorescence is characteristic of the adsorbate and not of the adsorbed substance itself. The same compound can show different kinds of fluorescence, according to the nature of the column ; this is particularly so when there are various opportunities for the formation of salts. The nature of the solvent may also have an influence.

A good example of this is furnished by the investigation of Carlsohn and Müller (1) into benzanthrone. This gives a golden-yellow solution in benzene. The fluorescence of its adsorbate on calcium carbonate, alumina or talc is canary yellow ; on natural bleaching earths it shows a green fluorescence, pale to bright, whereas on artificial bleaching earths, the colour is golden-yellow to reddish-orange. Owing to formation of salts with the complex acid earth, the last-mentioned fluorescence approaches the colour of that observed with a sulphuric acid solution. Artificially activated clays give rise to a fluorescence that appears, according to its content of loosely held adsorbed water, from red-orange to golden-yellow. Strongly adsorbed solvents, such as alcohol, displace benzanthrone from the surface of the clay ; this leads to a change of the yellow, green or orange fluorescence into blue.

Sometimes the presence of a substance has an astonishingly marked effect on the fluorescence colour of another. Thus naphthalene at a concentration of $\frac{1}{30000}$ per cent completely suppresses the fluorescence of anthracene ; a concentration of $\frac{1}{100000}$ per cent almost does so (Winterstein, Schön and Vetter).

If operations are carried out in aqueous solution or solutions containing water, the intensity and colour of the fluorescence may be markedly influenced by the prevailing pH. The

behaviour of the fluorescence-colour when hydrogen ion concentration is deliberately shifted can be typical for a particular substance and can distinguish it from substances that accompany and otherwise resemble it. A pretty example of this is shown in Koschara's (5) studies of uropterin: its behaviour is given in Table 9.

Clearly the domain of fluorescence chromatography is capable of further exploitation. In particular, many qualitative tests in analysis and industrial control, in which ultraviolet radiations have already been used, could, with advantage, be carried out on the Tswett column.

TABLE 9

EFFECT OF HYDROGEN-ION CONCENTRATION ON FLUORESCENCE OF UROPTERIN UNDER THE QUARTZ-MERCURY LAMP

Medium	Colour in Daylight	Fluorescence (U.V.) Colour
Strong mineral acid . .	Colourless	Red
Normal mineral acid . .	,,	Extinguished
Acetic acid	Yellow	Extinguished (dull yellow)
pH = 4	Greenish-blue	Yellow-green (weak)
pH = 7 to 11	,, ,,	Sky blue
Sodium hydroxide solution	Bluish-green	Green
Strong caustic soda solution	Yellow	Extinguished (moss green)

Dhéré, as well as Haitinger and also Danckwortt, have summarised information about the fluorescence phenomena of various classes of substance.

Fluorescence can also be spectroscopically investigated and several fluorescent zones can be examined at the same time, as Almasy has shown in the work mentioned below (spectrochromatography). The work was carried out with fractions containing 1 : 2-benzpyrene, prepared by high vacuum fractionation of coal-tar distillation residues.

The condensate containing benzpyrene is dissolved in hexane, adsorbed on alumina (Brockmann's; 15 to 20 by 0·8 cm.) and developed with hexane until no more fluorescent substances pass into the filtrate. Light from the quartz-mercury vapour lamp (with Schott filters UG2) then reveals

a number of different fluorescing zones. The tube, which is 30 cm. long, is made of completely transparent silica; it is set up in the manner shown in Fig. 35, near the slit of a Hilger quartz spectrograph (Type E2)—at about 120 cm. distance. The light from the heavily shaded lamp passes through a Wood's glass filter (Schott's No. UG2) and also a layer 8 mm. thick of 0·17 per cent salicylic acid solution. The fluorescent column is focussed on the slit of the spectrograph and the image must not be greater than the effective height of the slit (2 cm.). With a slit width of 0·05 mm., exposures of 15 to 30 minutes will give a number of adjacent-lying fluorescent spectra of various heights, corresponding with the individual zones of the chromatogram. To identify the benzpyrene, a fluorescence spectrogram of the adsorbed compound is used for comparison. (If some black rings are painted round the adsorption tube with lacquer, small clearly demarcated gaps appear on the chromatogram. From the disposition of the rings, that is, of the gaps, the scale of reduction can be ascertained and used to fix the final cutting up of the column.)

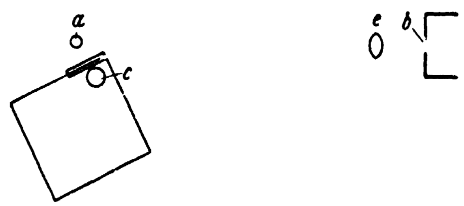


FIG. 35.—Lay-out for spectrochromatogram (plan): *a*, adsorption-tube; *b*, spectrograph slit; *c*, quartz-mercury lamp; *e*, quartz lens (Almasy).

5. Colour Reactions as a means of making a Chromatogram Visible

There are in the literature a number of examples of the fact that differentiation of material into invisible layers can be established by means of a colour reaction. This can be undertaken after the adsorbent has been more or less empirically cut, that is to say on portions of the eluate; it then falls, strictly speaking, outside the scope of chromatography. Alternatively it can be undertaken during the experiment itself, preferably on the column.

The first procedure was early exemplified by Winterstein and Stein (1) and concerns the separation of cholesterol and ergosterol, or at least the enrichment of the former in the latter (p. 261). The cholesterol mostly passed into the filtrate; the

column was divided into four parts and eluted with a mixture of ether and methanol. A strongly positive Rosenheim-Tortelli-Jaffé reaction was only given by the contents of the uppermost layer.

Kögl, Haagen-Smit and Erxleben chromatographed their hetero-auxin preparation from benzene solution on calcium carbonate and developed with benzene and alcohol (p. 279). Part of the material passed through the adsorbent and gave no reaction with ferric chloride in hydrochloric acid solution. The column was then cut into six pieces, each was eluted with alcohol and the dissolved solids from each eluate were tested for the colour reaction mentioned; it was positive primarily with the third and fourth fractions.

The number of these examples could be multiplied. We shall, however, turn to the other procedures, wherein the column is cut after the colour reaction has been carried out or is not cut at all. In the latter event the portions of a fluid chromatogram are tested by means of a colour reaction, the results of which can point the way to the differentiation of the individual fractions.

Ruggli and Jensen (2) succeeded in separating naphthol-sulphonic acids by means of an invisible chromatogram, prepared on alumina from aqueous solutions; this was developed with water, so that the individual zones appeared consecutively in the filtrate. They were collected in separate receivers and treated with a solution of diazotised Fast Red JTR-Base. The sodium salt of 1-naphthol-4-sulphonic acid gave a red, that of 2-naphthol-4-sulphonic acid a violet dye. In another series of experiments, the extruded column was treated with the diazo solution, which caused the upper half to turn violet and the lower red.

In many instances the procedure can be carried out by the following "brush method"—often without appreciable loss of material (Zechmeister, Cholnoky and Ujhelyi; Fig. 69, p. 323).

After the invisible chromatogram has been prepared, the column is extruded: a camel-hair brush that has been dipped into a suitable reagent is then drawn down the side of the column, parallel with its long axis, so as to make a narrow "streak." This "streak" will be sharply defined wherever it passes over a substance that can react to form a pigment.

With coloured reagents, such as permanganate, a change of tint may be observed. After the position of the required zone has been thus found and marked, the thin layer of adsorbent that has been moistened with the reagent is shaved off with a knife and the column is cut in the usual way.

Examples: (*a*) Separation of benzidine and α-naphthylamine. Every 30 mg. of the material is dissolved in a mixture of benzene, 20 ml., and light petroleum, 30 ml.: the solution is poured on to Brockmann's alumina. The reagent is an aqueous solution of sulphanilic acid and sodium nitrite; this evokes a green colour in the upper part of the column (benzidine). The naphthylamine is in the lower part and yields a red dye. A solution of lead peroxide in 30 per cent acetic acid may replace the diazo reagent; it gives a "streak" showing blue at the top and green below.

(*b*) Separation of α- and β-naphthol (in benzene and petroleum mixture, on alumina) is easily carried out by means of the diazo solution. The upper layer, β-naphthol, gives a light orange colour, the lower a violet colour.

(*c*) Permanganate can often be used; it served, for example, to localise ω-nitrostyrene (on calcium hydroxide, from petroleum) in the presence of saturated compounds.

(*d*) The position of some aldehydes may be established with Schiff's reagent, which gives a red colour with, for example, *m*-nitrobenzaldehyde adsorbed on calcium hydroxide from benzene and petroleum.

(*e*) Dimethyl glyoxime can be dissolved in a large quantity of warm benzene from which it is deposited on the upper portion of a column of calcium carbonate; it can be located there by the production of a red colour with nickel sulphate.

(*f*) Vitamin A preparations can be worked up on a calcium hydroxide column by making use of the Carr-Price reaction. (Brush dipped in chloroform solution of antimony trichloride, to give a blue colour.) (Fig. 69, p. 323.)

In exceptional instances, the brush technique gives rise to a streak that, though colourless, has ultra-violet fluorescence, which can be evoked under the quartz-mercury lamp. This method of procedure was recognised by Potts and Koch, who, however, did not actually use a brush: they cut a channel in the column, and allowed the reagent to run into it from a

pipette. Columns of soft materials will not stand up to this treatment.

For means of making visible inorganic adsorbates on an alumina column, see page 304. A remarkable instance is the coloration of a silver chloride zone, following illumination of the column, according to Schwab and Dattler. In our experience, the brush procedure can also be used in inorganic chromatography.

Note to 1st English Edition.—The following information about varieties of alumina obtainable in England may be of use to British chemists. Dr. A. H. Cook (private communication) has examined a representative sample of "Mayfair A" Brand Aluminium Oxide, marketed by Messrs. Savory & Moore Ltd., Wigmore Street, London, W.1. He finds it to be in almost all respects the equal of Merck's "standardised" material, and, indeed, in some respects superior. For general purposes, however, this brand, like the Merck variety, is too expensive. As an alternative, a very satisfactory product has been found both by Dr. Cook, and by our colleague Dr. E. Lester Smith, in the Birlec Activated Alumina (passing 100 mesh, not passing 120 mesh), produced by Birmingham Electric Furnaces Ltd., Tyburn Road, Birmingham, 24 ; it is also supplied by several of the leading dealers in chemicals and apparatus. It is slightly buff-pink in colour when moist, and a little more alkaline than the Merck or the Savory & Moore product.—TRANSLATORS.

SPECIAL SECTION

CHAPTER 3

APPLICATION TO NATURALLY OCCURRING PIGMENTS

"Our minds seem naturally inclined to devote special attention to all coloured substances."

TSWETT

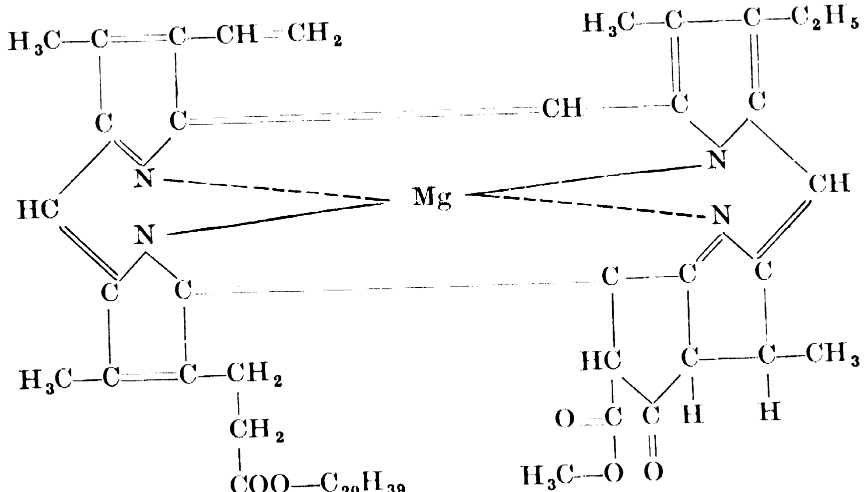
1. CHLOROPHYLL

Reference has already been made (p. 11) to Tswett's use of adsorption analysis in studying the pigments of green leaves. In 1911, Willstätter wrote, "The separation of the α- and β-forms (of chlorophyll) for purposes of spectroscopic examination is best effected by Tswett's method of chromatographic adsorption analysis." Willstätter and Isler, who themselves isolated chlorophylls *a* and *b* by another method, commented on the work of the Russian investigator: ". . . his data on absorption spectra are confirmed in all essentials by our own results. The chromatographic method, however, can only be used as yet on the small scale and would appear to be unsuitable for preparative work. How far Tswett succeeded in avoiding allomerisation of the chlorophyll during isolation of the components by chromatography is not yet clear; not spectroscopic examination, but only identification of phytochlorins and phytorhodins, is here decisive."

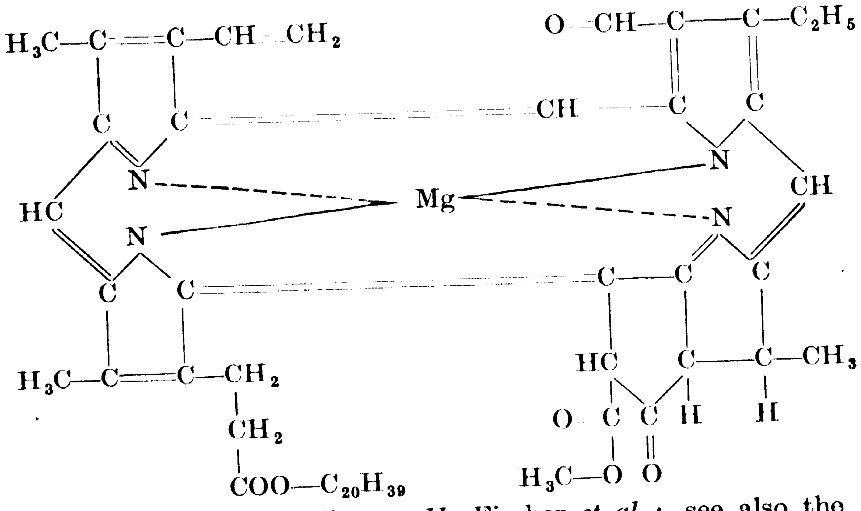
The fears thus expressed by Willstätter and Isler concerning the destruction of chlorophyll during adsorption were in part justified, for Winterstein and Stein (2) have shown that chlorophyll undergoes a change when adsorbed on calcium carbonate, alumina, fuller's earth, sodium sulphate or certain other adsorbents; the material recovered from such columns does not give the "phase test." Tswett used sugar (sucrose) as adsorbent and obtained two zones, a dark green at the top

of the column due to chlorophyll *b* and a blue below it due to chlorophyll *a*. In fact, chlorophyll undergoes little or no destruction by adsorption on sugar and Tswett's original method can be used satisfactorily for preparative work. Mackinney (3) has shown that chlorophyll also undergoes little decomposition when adsorbed on inulin. In this instance, dichloroethane was used as the solvent.

Winterstein and Stein (2) stated that, whereas the chlorophyll *b* of Willstätter and Stoll contained 15 to 20 per cent of



Chlorophyll *a* (according to H. Fischer *et al.*; see also the publications of A. Stoll).



Chlorophyll *b* (according to H. Fischer *et al.*; see also the publications of A. Stoll).

chlorophyll *a*, the chlorophyll *b* obtained by chromatography contained none.[1] On the other hand, Stoll and Wiedemann showed that the material prepared without using chromatography was substantially pure and this has since been confirmed by comparing material made by the two processes.

Zscheile has also made use of chromatography in his researches on chlorophyll, but with talc as adsorbent instead of sugar. He used a mixture of ether and light petroleum (b.p. 40° to 60° C.) as the solvent, and ether for elution. Zscheile obtained a third zone in addition to the zones corresponding to the two known components, and he gave the name chlorophyll *c* to the new substance that he believed to be responsible for this third zone. Winterstein and Schön (1) using sugar could find no evidence of the existence of this third component, and suggested that the third zone was due to a decomposition product formed by the action of the talc. Mackinney (3) using inulin also found no indication of the existence of chlorophyll *c*. Spoehr prefers inulin to sugar as an adsorbent for chlorophyll.

Estimation of the Relative Amounts of Chlorophylls *a* and *b* in a Mixture

The amounts of chlorophylls *a* and *b* in a mixture of the two can be determined by chromatographic separation followed by colorimetric estimation of the two fractions. Tswett himself emphasised that the widths of the bands on the column give no clue to the proportions of the two constituents, and estimates obtained in this way are apt to be misleading.

Winterstein and Stein (2) measured the proportion of the two constituents of chlorophyll, using only one or two leaves for the purpose. A length of fine wire was twisted round the leaves, which were then frozen in, for example, liquid nitrogen. They were then powdered as quickly as possible with a pestle, stirred with 25 ml. of a mixture (9 : 1) of petroleum (b.p. 70° C.) and benzene and stirred again after the addition of 8 ml. of methyl alcohol. The suspension was filtered and washed with the solvent mixture until the residue was colour-

[1] The figures given by Winterstein and Stein (2) must be too high; Willstätter and Stoll's old samples contain only minute traces of chlorophyll *a*.

less. The methyl alcohol was removed from the filtrate by washing cautiously with several quantities of water, vigorous shaking being avoided. The pigment solution was filtered through a dry folded filter, and then poured on to a column (10 × 1 cm.) of powdered sugar, and the chromatogram developed with a mixture 4 : 1, 9 : 1 or 19 : 1 of petroleum and benzene. The proportion of petroleum to benzene was varied according to the rate at which the zones were found to move down the column. The mixture containing the most benzene developed the column most rapidly. If development proceeded too quickly, forming broad streaky zones, light petroleum (b.p. 30° to 50° C.) was substituted for the mixture of petroleum and benzene.

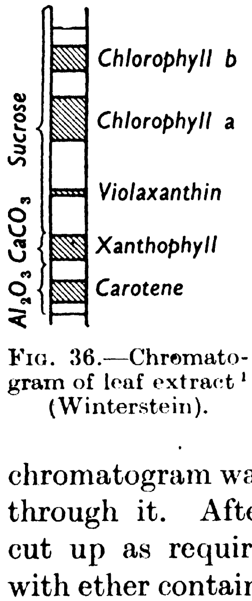


FIG. 36.—Chromatogram of leaf extract[1] (Winterstein).

Carotene passes right through such a column and can be estimated colorimetrically in the filtrate if desired. Xanthophyll also usually passes through the column. If, however, the column of sucrose has been prepared above a column of calcium carbonate, and this in turn above a column of alumina, the xanthophyll is retained on the calcium carbonate, and the carotene on the alumina (Fig. 36).

The petroleum and benzene were then washed out of the column by means of light petroleum (b.p. 30° to 50° C.) and the chromatogram was dried by drawing a current of carbon dioxide through it. After removal from the tube, the column was cut up as required and the portions were separately eluted with ether containing methyl alcohol. The eluates were washed free from methyl alcohol and concentrated to 15 ml. and the colour was measured in a step-photometer (filter S43) previously calibrated by means of solutions of pure chlorophylls *a* and *b*. The chlorophyll *b* solution should be examined spectroscopically for possible traces of chlorophyll *a*, and allowance made for any found to be present. In young clover leaves the ratio, chlorophyll *b* : chlorophyll *a*, was found to be 1 : 2·96.

[1] The identity of "violaxanthin" is still uncertain.

Isolation of Chlorophylls *a* and *b* from Crude Chlorophyll

(Winterstein and Stein 2)

A column of powdered sugar was prepared as previously described (p. 64); 3 g. of crude chlorophyll containing 60 per cent of chlorophyll *a* and 40 per cent of chlorophyll *b* were dissolved in 100 ml. of thiophene-free benzene and the solution was diluted to 1500 ml. with petroleum (b.p. 70° C.). After saturating the column with 200 ml. of a mixture 14 : 1 of petroleum and benzene the chlorophyll solution was poured on to it, followed by 150 ml. of the petroleum-benzene mixture. The chromatogram was developed with petroleum (b.p. 70° C.); the column was finally washed through with light petroleum (b.p. 40° to 60° C.) and sucked dry. (Time required, 1–1½ hours.)

The column was divided into three parts. The upper portion contained chlorophyll *b* (90 per cent pure), the second portion contained a mixture of chlorophylls *a* and *b*, and the third pure chlorophyll *a*. Each portion was separately stirred with 1 litre (1 : 1) of a mixture of acetone and peroxide-free ether and filtered, and the sugar was washed with the same solvent mixture until colourless. The acetone was removed from the filtrate by careful washing with water, vigorous shaking being avoided, and the ethereal solution was then poured through a filter containing a small amount of anhydrous sodium sulphate. The filtrate was distilled under reduced pressure at 25° C. and the residue was dissolved in a little ether and filtered; the filtrate was concentrated to a small bulk under reduced pressure. Finally the chlorophyll was precipitated by adding light petroleum. The suspension was centrifuged, the supernatant liquid was decanted and the precipitate was re-dissolved in a little ether. The solvent was then evaporated off completely and the resulting chlorophyll was dried in a vacuum. By this method 0·7 g. of pure chlorophyll *a* and 1·3 g. of chlorophyll *b* of 90 per cent purity were obtained.

In order to prepare pure chlorophyll *b* from the 90 per cent material, 0·2 g. was dissolved in 15 ml. of benzene and diluted to 150 ml. with petroleum (b.p. 70° C.). This solution was poured on to a column of sugar (12 × 5·5 cm.) and the

chromatogram was developed with petroleum-benzene mixture (14 : 1). It was washed with light petroleum (b.p. 40° to 60° C.) and sucked dry, and the column was divided up as before. Each portion was separately eluted, the eluates being treated as described above. For purification, the product from four or five experiments was dissolved in a little ether and precipitated by the addition of light petroleum. The precipitate was centrifuged off and the process was repeated. After two or three such precipitations, pure chlorophyll *b* was obtained.

The separation of phæophytins *a* and *b* can also be carried out by chromatographic adsorption, although these degradation products are not so strongly retained by sugar as are the chlorophylls. A mixture of sugar and talc is better than sugar alone.

Bacteriochlorophyll

The chromatographic method can be applied to the examination of bacteriochlorophyll. Gaffron (2) successively extracted red sulphur bacteria on a column of talc with alcohol, acetone and ether, and observed the formation of a green, a yellow and a red zone. The green pigment is bacteriochlorophyll, the degradation products of which were studied by Fischer and Hasenkamp (1) (see p. 96).

Animal Chlorophyll

The method of Tswett was employed in this field as early as 1916, when Dhéré and Vegezzi (see also Vegezzi) investigated the liver pigments of the Roman snail (*Helix pomatia*) by this method. A petroleum extract was poured on to a column of calcium carbonate ; when this was developed, four distinct zones were formed :

Top :	Yellow band. Xanthophylls.
	Double green band.
	Grey zone.
Bottom :	Yellow band. Carotene (passed into the filtrate).

The investigation showed that the chlorophyll obtained by the animal from the vegetation on which it feeds had been converted, probably by the acids of the digestive tract, into phæophytins *a* and *b*, or substances closely resembling them.

These degradation products were concentrated in the middle green zone of the chromatogram and occurred below the xanthophyll zone; chlorophyll would have been adsorbed above the xanthophyll zone.

Derivatives and Degradation Products of Chlorophyll. Precursor of Chlorophyll

The interesting question whether both chlorophylls *a* and *b* are derived from one common naturally-occurring proto-chlorophyll, from which they are formed by oxidation, or whether the plant contains a mixture of two proto-chlorophylls, was decided by Seybold in favour of the latter alternative. The seeds of a species of gourd were used in the experiment. The green cotyledons were dissected out from the seeds and extracted with a mixture of petroleum (b.p. 70° C.) and methyl alcohol; the alcohol was removed by washing with water and the petroleum extract was then concentrated and chromatographed on a column of powdered sugar. As with crude chlorophyll, a green zone was formed at the top of the column and a yellow feebly-adsorbed zone of carotenoid below it. The column was washed ten times with petroleum (b.p. 70° C.) to remove the fat, and it was then developed with benzene or a mixture of benzene and petroleum. The original green band was split up by this treatment into a series of bands, a yellowish-green band at the top, then a bluish-green band, and a yellow carotenoid band below this. The two green bands were separately eluted with a mixture of methyl alcohol and ether, and the alcohol was removed by washing with water. The colours of the ethereal solutions, like those of chlorophylls *a* and *b*, were different, so that there appear to be two proto-chlorophylls. The fluorescence of the *b* component was weaker than that of the *a* form and the absorption band with the highest maximum occurred at a longer wave-length. The positions of the absorption bands of the two forms differ so little, however, that one cannot thus distinguish between the individual proto-chlorophylls and a mixture of the two. The existence of the two precursors of chlorophyll could not have been demonstrated, therefore, except by Tswett's method.

Reversible Reduction

Kuhn and Winterstein (2) converted chlorophylls *a* and *b* into the corresponding leuco-compounds by adding zinc dust and a little acetic acid to their pyridine solutions. Exposure to the air rapidly turned the brown filtrate green, and the regenerated chlorophyll was separated from the decomposition products that accompanied it, as follows.

The filtrate was first diluted with petroleum and the pyridine was completely removed by washing with water. The solution was chromatographed on a column of powdered sucrose and the chromatogram was then developed with petroleum. Three zones were obtained; with the product from chlorophyll *a*, for example, the column had the following appearance:

Top:	Narrow black band. Unknown decomposition product.
	Blue band. Chlorophyll *a* (70–80 per cent of the crude pigment).
Bottom:	Pale grey band. Apparently phæophytin.

The reaction mixture from chlorophyll *b* gave a greenish-yellow band in the upper portion of the column in place of the blue band due to chlorophyll *a*.

Chromatography has been extensively employed by H. Fischer and his co-workers, and the following examples demonstrate some of its applications.

Bacteriophæophytin

Fischer and Hasenkamp (1) triturated the purple-coloured mass of sulphur bacteria (from 150 litres of culture solution) with acetone and, after allowing the mixture to stand for a day, extracted it three times with 80 per cent acetone. The extract was poured into ether and the ethereal solution was washed several times with distilled water and finally shaken with 10 per cent hydrochloric acid. The ethereal solution was concentrated to 20 ml. and the precipitated bacteriophæophytin was filtered off. The mother liquors were poured on to a column of talc, which was then developed with acetone. A small quantity of phorbide-like compound (? *b* series) was thereby obtained; it differed spectroscopically from the main component.

Gaffron (1) prepared bacteriophæophytin on a smaller scale.

Experiments with Phorbides

(*a*) Using a column of talc, Fischer and Hasenkamp (2) freed dihydrophæophorbide *a* from the phæophorbide *a* responsible for the "oxo-reaction" previously described by them. The chromatogram was prepared by pouring a saturated ethereal solution on to the column, which was developed by means of a mixture of acetone and ether. The phæophorbide *a* was adsorbed on the upper part of the column.

(*b*) Fischer and Stadler chromatographed the "probophorbide" obtained from the fresh fæces of sheep. A solution in acetone was poured on to a column of talc and the chromatogram was developed with ether. Two coloured zones were obtained, of which the lower contained dihydropyrophæophorbide *a*. The heterogeneous nature of the so-called "probophorbide" was thus established by chromatography.

(*c*) Fischer and Schmidt used chromatography to purify phæophorbide *a*-geranyl ester, $C_{45}H_{52}O_5N_4$.

(*d*) Fischer and Medick worked up by the chromatographic method the reaction-mixture produced by the action of diazoacetic ester on methyl phæophorbide *a*. The substance was dissolved in a mixture of acetone and ether, and light petroleum (b.p. 30° to 50° C.) was added to the point of precipitation. This solution was poured on to a column of talc and the chromatogram was developed with a mixture (5 : 2) of ether and light petroleum (b.p. 30° to 50° C.). This produced a yellowish-green zone at the top of the column, a yellowish-grey zone below this, and lowest of all a grey zone; on elution with ether this last portion yielded a small quantity of a crystalline rearrangement product, $C_{39}H_{42}O_7N_4$.

(*e*) In the conversion of phæophorbide *b* into the corresponding chlorophyllide (by means of the Grignard reaction), Fischer and Spielberger first washed the pyridine-ether solution of phæophorbide (0·5 g.) free from pyridine, concentrated the ethereal solution to 70 ml. and poured this on to a column of talc (8 × 5 cm.) prepared with a mixture of ether and light petroleum (b.p. 30° to 50° C.). From the filtrate, 3 mg. of crystalline chlorophyllide were obtained on dilution with water whereas no crystalline material was obtained from the column after elution with acetone-pyridine.

(*f*) In order to purify the crude 2-α-hydroxy-meso-chlorin-e_6-trimethylester obtained by the addition of hydrogen bromide to chlorin-e_6-trimethylester, followed by hydrolysis and esterification, Fischer, Lautsch and Lin dissolved 2 g. of the crude material in the least possible quantity of methyl alcohol and chromatographed the solution on a column of talc (40–50 × 2–3 cm.). The column was prepared and developed with methyl alcohol. Yellow decomposition-products were adsorbed on the column ; the deep-blue filtrate was evaporated under reduced pressure and the residue was dissolved in glacial acetic acid. Light petroleum (b.p. 30° to 50° C.) was added until crystallisation commenced. After four recrystallisations, the substance, which formed blue prisms, had a melting-point of 215° C.

(See also Fischer and Laubereau.)

2. Hæmin

Barkan and Schales (2) obtained a water-soluble hæmin by the action of hydrochloric acid, with or without digestion, on blood of various kinds. With pyridine, nicotine and other nitrogenous bases, this hæmin gives hæmochromogens showing the spectrum of cytochrome *c* ; for this reason it is called *c*-hæmin. According to Schales (1, 2) the new kind of hæmin is prepared in the following manner.

A solution of crystalline oxyhæmoglobin from horse's blood, containing 4·5 per cent of pigment in 300 ml., was incubated for 48 hours at 38° C. with a solution of 0·6 g. of Witte's pepsin in 300 ml. of 0·8 per cent hydrochloric acid. The liquid was extracted in a separating funnel with ether, water being added to facilitate the separation. The lower layer was tapped off, and the brown precipitate that collected at the interface was rejected. The aqueous layer was re-extracted with three further portions of ether, the final extract being colourless. The aqueous solution, measuring 1·3 litres, was freed from ether by warming on the steam-bath, cooled, and filtered. The solution, now free from protohæmin, was exactly neutralised with potassium carbonate solution and evaporated to dryness under reduced pressure, and the residue (11·7 g.) was dried over phosphorus pentoxide.

A mixture of 20 ml. of M/15 phosphate-buffer solution (*p*H 8), 4 ml. of pyridine, and a little sodium hydrosulphite solution, was added to a solution of 8·3 g. of the buff-coloured product in 60 ml. of water. The reddish solution so obtained was drawn slowly through a column (16 × 3 cm.) of Brockmann alumina. After about three-quarters of an hour all the liquid had passed through the column, which then had the following appearance: a sharply-defined deep-red ring 1 to 2 mm. wide occurred some 1·5 cm. from the top of the column, with a yellow to brown zone beneath it; below this, and about 6 cm. from the top of the column, was an ill-defined yellowish-green band, followed by a reddish-brown and then a yellow zone. On developing the column with 25 ml. of the solution containing sodium hydrosulphite, the region around the red band became colourless; the filtrate was yellow to bright green, and was free from *c*-hæmin. The column was sucked as dry as possible and the red band, which turned brown on exposure to air, was eluted with 2/3 N-hydrochloric acid; the brown solution was exactly neutralised with sodium hydroxide solution. Brown flakes formed; after standing for a short time, these were centrifuged off, washed with acetone and ether, and dried in a vacuum desiccator over phosphorus pentoxide. The yield was 0·2 g. of *c*-hæmin in the form of a dark brown powder. A little of the substance was dissolved in hydrochloric acid, the solution was made alkaline with sodium hydroxide solution, pyridine was added, and the *c*-hæmin was reduced with sodium hydrosulphite. The resulting solution gave adsorption bands at 553·8 to 545·6 and 519·1 mμ.

3. Porphyrins

The Porphyrins of Urine

Normal Urine

The colour of normal urine, according to Waldenström, is not reduced by filtration through a column of alumina, but an alcoholic solution of urochrome forms a brownish-yellow zone on such a column. The urochrome can be eluted by means of water.

Coproporphyrin I, which is present in normal urine at a dilu-

tion of 1 : 10^8, was concentrated by Fink (1, 2) by adsorptive filtration through a mixture of cotton fibre and asbestos. The solution employed had a pH of 3·6, and under these conditions the colloidal porphyrin possessed a positive charge, the filter being negatively charged (see also Hoerburger). Zeile and Rau obtained this same coproporphyrin from urine by the method of Waldenström described below.

Pathological Urine

Chromatography was used by Waldenström (1, 2) when investigating the red pigments that occur in relatively large amounts in the urine of patients suffering from such disorders as porphyrinuria and lead poisoning. " Aluminium oxydatum anhydr. puriss. Merck " was used, and the normal pigments of urine passed through the column into the filtrate, whilst the porphyrins and other pigments of pathological origin were adsorbed by the alumina. This method is thus well adapted for concentrating such pigments, and their further purification can be carried out according to established methods.

The pigment was directly adsorbed from the urine, that is, from an aqueous solution. The column was not removed from the tube, and the pigment was recovered from it by treatment with a suitable eluting agent.

Apparatus : To a 1-litre separating funnel was sealed a glass tube 20 × 3·5 cm. carrying a stop-cock with a bore of 0·8 cm. A plug of damp cotton-wool was forced into the hole. The adsorbent was poured into the tube to a height of 5 to 10 cm. and saturated with water. The apparatus was fixed into a filter-flask and gentle suction was applied until only a 1-cm. layer of water covered the adsorbent. After addition of 1 to 2 per cent of acetic acid to the urine, it was poured on to the column down a glass rod, which almost touched the adsorbent to avoid disturbing the alumina. The stop-cock was then opened and the liquid was allowed to run through, gentle suction being applied if necessary.

Example : A patient suffering from acute porphyria excreted urine that reacted neutral, was deep red in colour, and gave a negative aldehyde reaction (Ehrlich). The urine was acidified with acetic acid and extracted with ether, whereby some coproporphyrin passed into solution. The aqueous layer was

filtered from the precipitate simultaneously formed, and the filtrate was chromatographed as described above. At first a reddish-brown zone appeared in the upper portion of the column, but this moved slowly downwards. The column immediately below this zone was coloured yellowish-brown, whilst the uppermost portion gradually became dark-red—almost black—in colour. The column was washed with 1 per cent acetic acid and then with 20 per cent acetic acid. This first brought about elution of the yellowish-brown region, and then of the reddish-brown band; both eluates were collected separately and were porphyrin-free. The column was washed with further quantities of acetic acid and all the pigment except the uppermost zone was thereby eluted; this fraction contained porphyrin. After a further washing with water, the remainder of the pigment was removed with 12 per cent ammonia solution, giving a deep-red solution; the alumina was completely decolorised. The ammoniacal solution contained large amounts of porphyrins. As these were not precipitated on making the solution slightly acid with acetic acid, the chromatographic adsorption was repeated. Precipitation of the pigments was obtained on adding acetic acid to the ammoniacal solution obtained from this second chromatogram. Precipitation does not appear to occur after a single chromatographic treatment, and the isolation of the pigment has been accomplished only by adsorption as described above. The urine after the treatment is bright yellow in colour, and this provides an easy check on the porphyrin content. The method can be used for small quantities of urine (10 ml.) and for samples of urine containing only small amounts of porphyrin.

It is well known that the red colour of urine in cases of acute porphyria is partly due to substances that accompany the porphyrins, but are not themselves porphyrins. In order to examine these as yet unknown pigments, the urine was adsorbed as described above. The porphyrins were held at the top of the column and the other pigments below them, forming a red zone that moved rapidly downwards and ultimately passed through the column into the filtrate, which acquired a deep-red colour, but was nevertheless porphyrin-free. The filtrate was chromatographed and the column was washed, first with methyl alcohol, then with methyl alcoholic ammonia

solution. The pigment was eluted from the column with very dilute ammonia solution.

Detection of two Isomeric Uroporphyrins in the Urine of a Patient suffering from Congenital Porphyria

(Fischer and Hofmann).

A uroporphyrin preparation (Petry) of melting-point 286° Cl was dissolved in the least possible amount of chloroform-methy. alcohol mixture (1 : 1) and the solution was poured on to a column of talc prepared with the same solvent. The single pink zone at first formed separated on development with chloroform into an upper narrow, brownish-red band and a lower pink band. The spectra of both of these substances dissolved in a mixture of pyridine and ether were identical. The lower band was washed through into the filtrate, which crystallised on concentration, yielding needles of melting-point 300° to 302° C. (uncorr.). Since a second chromatogram gave no further separation, this substance, uroporphyrin I, was considered to be homogeneous.

The upper zone, containing uroporphyrin III, was washed with chloroform-methyl alcohol mixture (7 : 3) to remove other substances, and the reddish-brown portion of the column (8 cm.) was then shaken with glacial acetic acid for some time, the resulting solution was diluted with 3 volumes of water, and the porphyrin was quantitatively extracted with ethyl acetate. The extract was washed free from acid, dried, concentrated to a volume of 4 ml., centrifuged and decanted, and again concentrated. The solution deposited needles that had a melting-point, as well as a mixed melting-point with an authentic specimen of uroporphyrin III (Waldenström), of 257° C.

Using the method of Waldenström (see above) Turner has detected porphyrin of type I in the normal urine of the black squirrel (*Sciurus niger*).

Porphyrins from Fæces

Waldenström extracted the dried, finely-ground fæces obtained from a patient (Petry) with glacial acetic acid-ether mixture, transferred the porphyrin to hydrochloric acid, and

neutralised the solution to congo red with sodium acetate. The solution was chromatographed on a column of alumina. A yellow filtrate was obtained, the porphyrin remaining on the column. After being washed with water and then with ether, the column was treated with glacial acetic acid. The porphyrin was thereby eluted and to the eluate were added several volumes of ether. The acetic acid was removed from the ethereal solution by washing it with water, and the porphyrin was readsorbed on a column of alumina. By developing the column with ether containing 1 per cent of acetic acid, a proper chromatogram was obtained. First a pale green zone, formed by a coproporphyrin, migrated down the column, and then the portion containing the bulk of the porphyrin.

Removal of Protoporphyrin from the Porphyrin of Fæces

Grotepass and Defalque extracted the fæces obtained in a case of porphyria without porphyrinuria in the usual way and transferred the porphyrin from the acetic acid-ether mixture to 5 per cent hydrochloric acid. This solution was filtered through a layer of talc 2 mm. thick, which was then washed with 10 per cent hydrochloric acid until the filtrate was colourless. Other pigments present as impurities remained adsorbed on the talc. The red filtrate was purified by extraction three times with ether. A column (15 cm.) of infusorial earth, freed from iron by repeated washing with warm 5 per cent hydrochloric acid, was prepared, and the hydrochloric acid solution of porphyrin was poured on to it. A brown band 3 cm. wide formed at the top of the column, which was washed with 5 per cent hydrochloric acid until the filtrate, at first red, became quite colourless. The filtration through a column of infusorial earth was repeated twice more in order to remove the last traces of protoporphyrin, as shown by spectroscopic examination of the acetic acid-ether solution. Other porphyrins present in the solution were separated from one another by known methods.

Synthetic Porphyrins

(*a*) Synthetic coproporphyrins I and III can be separated by Waldenström's method.

(*b*) Synthesis of desoxo - phyllerythroætioporphyrin,

$C_{32}H_{36}N_2$ (Fischer and Hofmann 1). This compound was isolated from the porphyrin mixture, in the following manner. As concentrated a solution as possible (3 g. in 15 litres) of the reaction mixture in ether was prepared and run slowly for a period of several days through a conical tube (70 × 12 cm.) filled with talc. The whole of the porphyrin was adsorbed in the upper third of the column, which was developed with a mixture (10 : 1) of ether and chloroform. The ætioporphyrin was retained in the upper part. This was removed and eluted by digestion with large amounts of pyridine. The eluate was evaporated under reduced pressure and the residue was dissolved in the smallest amount of ether. This solution was chromatographed, whereby the remaining ætioporphyrin was removed; between the two bands the column was perfectly white. The upper band was eluted with pyridine, the eluate was concentrated, the residue was taken up in ether and the pyridine was washed out of the ethereal solution with water. Fine needles separated out on concentrating the solution.

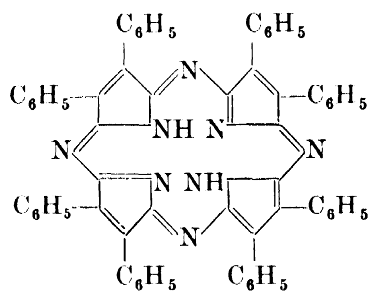
(*c*) Purification of phylloporphyrin ester, obtained by formic acid degradation of phytochlorin (Fischer and Bauer). A column of talc was used and was eluted with a mixture of pyridine and ether.

(*d*) Ferrous salt of tetrabenzo - monoazaporphin, $C_{35}H_{19}N_5Fe$. The pyridine mother-liquors remaining from the isolation of the diaza compound, $C_{34}H_{18}N_6Fe$, obtained by interaction of *o*-cyano-acetophenone and iron powder (Helberger, Rebay and Hever), were diluted with ether and poured on to a column of alumina. The chromatogram was developed with ether containing 5 per cent of pyridine. Three streaky greenish-brown bands close together occupied the upper part of the column. Below these bands was a broad zone formed of three bands overlapping one another; it was blue at the top and violet at the bottom. This part of the column contained the diaza-compound at the top, the monoaza compound in the middle and the iron salt of tetrabenzoporphin, $C_{36}H_{20}N_4Fe$, at the bottom. As the washing was continued, the bands were washed one by one into the filtrate and the receiver was changed whenever a new absorption band made its appearance in the red region of the spectrum. Thus fraction 1 had a band at

600 mμ, fraction 2 two bands at 620 and 600 mμ, and fraction 3 three bands at 650, 620 and 600 mμ. The middle fraction was concentrated and chromatographed again. Fractions 1 and 2 were obtained, together with a third fraction that showed only weak absorption at 600 mμ. This was concentrated, and the residue was treated with hot methyl alcohol, from which large rhombic violet-coloured crystals were obtained. These consisted of a compound of the monoaza-derivative with 2 molecules of pyridine.

The magnesium compound of tetrabenzoporphin was similarly purified on a column of alumina, this being developed with a mixture (1 : 4) of pyridine and ether.

(*e*) Porphyrazins. By heating diphenyl maleic nitrile, $(C_6H_5)(CN)C=C(CN)(C_6H_5)$, with magnesium, Cook and Linstead obtained magnesium-octaphenyl porphyrazin. A solution of the purplish-black powder (0·6 g.) in 300 ml. of benzene was chromatographed on a column (20 × 1·5 cm.) of Brockmann alumina. There was formed a bright green ring and a brown zone containing a small quantity of impurity.



Octaphenyl-porphyrazin

The main band was eluted with pyridine and the concentrated eluate was treated with water. The free octaphenyl porphyrazin obtained by treatment with hydrochloric acid was purified in the same way. It was less strongly adsorbed than were the traces of the magnesium compound that remained. When copper bronze was used instead of magnesium, the corresponding copper-compound was obtained ; after purification in a similar manner, it was isolated in crystalline form.

4. Bile Pigments

Chromatography has as yet found little application in this field.

(*a*) Photochemical transformation of ætio-porphyrin I into bilirubinoid pigments (Fischer and Herrle)

A solution of ætioporphyrin (2 g.) in pyridine (4 litres) was mixed with 10 per cent sodium ethylate solution until all the material was present as complex salt, showing absorption bands at 591·4, 555·5, 445·3 mμ. The same amount of sodium ethylate solution was then added and the mixture was passed through a glass spiral coiled round a 500-watt tubular lamp. The red solution changed first to a blue and then to a deep-green colour. It was concentrated and poured into 3 litres of ether. Unchanged ætioporphyrin was extracted from this solution by 7 per cent hydrochloric acid and a second porphyrin by 12–15 per cent hydrochloric acid. This new compound was adsorbed on alumina and the chromatogram was developed with ether ; it had the appearance shown in Fig. 37.

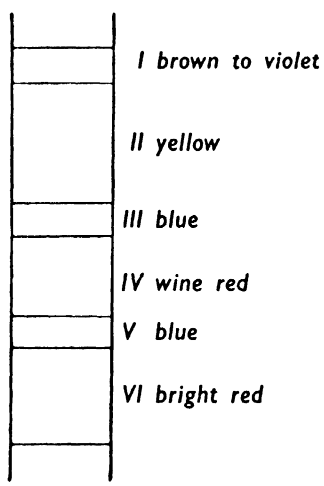
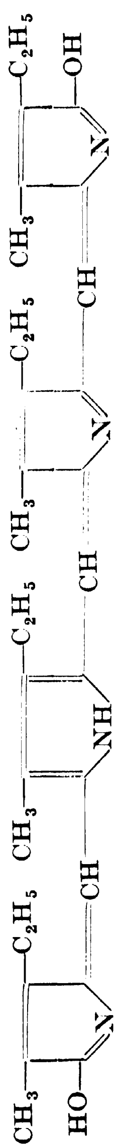


FIG. 37. — Chromatogram of the Irradiation Product of Ætio-glaucobilin or Ætio-porphyrin I.

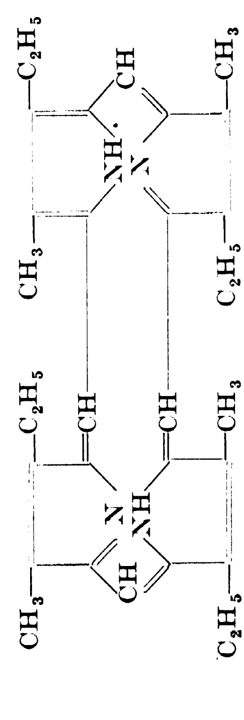
Zone II contained a new compound, $C_{16}H_{20}O_2N_2$, which was isolated by re-chromatographing the eluate from this zone. Ætio-glaucobilin, $C_{31}H_{38}O_2N_4$, was present in zone III, from which it was isolated by extraction with ether and evaporation of the solution ; it formed crystals of melting-point 238° C. From zone IV a red ketone, $C_{31}H_{36}O_3N_4$, was obtained after re-chromatographing ; it had a melting-point of 244° C. This compound is possibly identical with ætiomesobilirubinogen.

(*b*) Properties of the Bilirubin of Blood-serum. Separation of the Carotenoids of Serum

Süllmann, Szécsényi-Nagy and Verzár extracted an acidified aqueous-alcoholic solution of bilirubin with light petroleum and



Ætio-glaucobilin



Ætio-porphyrin I

adsorbed the pigment from this solution on anhydrous sodium sulphate. Bilirubin is more strongly adsorbed than lutein, and the filtrate was colourless. No pigment was adsorbed, however, when serum extracts were chromatographed in this way, apparently because of the lipoids present. A similar result was obtained when cholesterol was added to light petroleum solutions of pure bilirubin and lutein.

Alumina was found to be more suitable, and this readily adsorbed bilirubin from alcoholic solution. The irradiation product of sodium bilirubinate was adsorbed even more readily. The following procedure was adopted to separate the bilirubin and the lipochromes of human blood-serum : 10 ml. of the serum were treated with 75 ml. of alcohol ; after standing for half an hour in the dark, the solution was filtered through a sintered-glass crucible (G_3). The precipitate was washed twice with alcohol, the second time after the addition of a little light petroleum. The filtrate was acidified and extracted with light petroleum, and the solution of serum pigments so obtained was chromatographed on alumina. The chromatogram showed the following zones :

Top : Yellow, not eluted by light petroleum, but eluted by alcohol, diazotisable : bilirubin.
Reddish-yellow, readily eluted by alcohol ; hypophasic on partitioning between light petroleum and 87 per cent alcohol : xanthophylls.
Bottom : Reddish-yellow, epiphasic : carotenes.

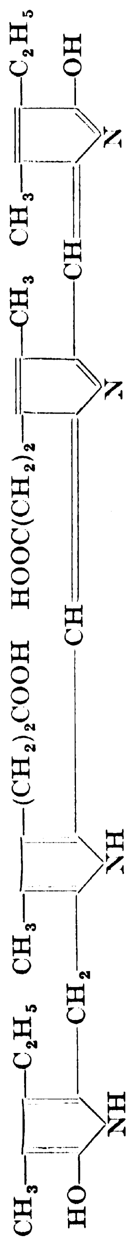
A more complete fractionation was not possible on account of the small quantity of serum used. There was sufficient material for a quantitative estimation, however.

(*c*) Urine

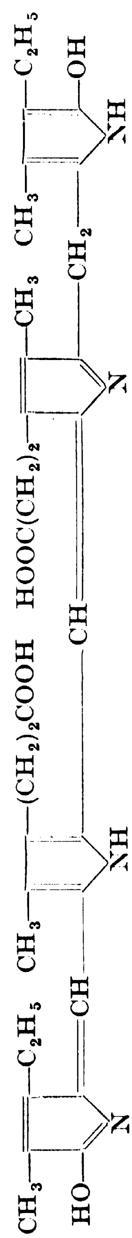
Urobilin, like urochrome (p. 99), is only adsorbed from the first few millilitres of urine when this, made slightly acid with acetic acid, is poured on to a column of alumina. Bilirubin and biliverdin, on the other hand, are readily adsorbed from urine containing bile-pigments (Waldenström).

(*d*) Degradation Products.

The pigment obtained from mesobilirubinogen by oxidation with ferric chloride was transferred to chloroform ; the solution was concentrated and mixed with an equal volume of



Top: Mesobilirhodin



Bottom: Mesobiliviolin

ether. The mixture was poured on to a column of talc and this was developed with a mixture (2 : 1) of chloroform and ether (Siedel). Two sharp zones were formed, the upper bright red, the lower violet in colour. The red pigment was termed mesobilirhodin and the violet pigment mesobiliviolin. The formulæ of these compounds show that even the smallest variation in structure can make a great difference to the behaviour of substances on a column.

(*e*) Synthetic Hexa-pyrrens

A substance termed "hexapyrren" was obtained by Fischer and Reinecke. The condensation product of neoxanthobilirubinic acid and 3 : 3′-dimethyl-4 : 4′-dipropionic acid-5 : 5′-dibromomethyl-pyrromethen-hydrobromide, a substance built according to the "bilirubin-urobilin-model" of these workers, underwent dehydration on standing in methyl alcoholic hydrogen bromide solution. The mixture was poured into chloroform, the solution was washed several times with water and the pigments were adsorbed on alumina. After development with acetone, the column had the following appearance :

Top :	Zone I	orange	
	Zone II	violet	
	Zone III	blue	no white zones between these bands
	Zone IV	violet	
Bottom :	Zone V	green	

The column was divided up and each part was separately eluted with methyl alcohol ; the pigments were re-chromatographed with a mixture of chloroform and acetone as solvent.

Zone I : The ethereal solution had an absorption band at 530 mμ. On adding ammonia and zinc acetate in alcoholic solution a reddish-violet colour was produced. This solution had two absorption bands, at 645 (weak) and 595 mμ (strong).

Zone II : The solution gave the violet Gmelin's reaction characteristic of a tetranuclear bilirubinoid compound. The 2 per cent hydrochloric acid solution was violet. With zinc acetate dissolved in alcohol a solution with absorption bands at 628, 576, and 515 mμ was obtained.

Zone III : This yielded crystals of melting-point 238° C. identical with the glaucobilin dimethyl ester obtained from

neoxantho-bilirubinic acid and formyl-neoxantho-bilirubinic acid.

Zone IV : The 15 per cent hydrochloric acid solution had a bluish-violet colour. This was due to the presence of a dehydrogenation product of the starting material.

Zone V : The solution was green and gave neither Gmelin's reaction nor the zinc acetate reaction.

When the chloroform solution of the hexapyrren (free base) was allowed to stand, it became violet in colour, owing to atmospheric oxidation ; when this solution was poured on to a column of alumina, it yielded two separate pigments, first an orange-yellow pigment that was very strongly adsorbed, and secondly a violet pigment that passed through the column into the filtrate. A column of alumina was also used in separating the reaction products obtained by dehydrogenation with hydroxylamine.

5. Carotenoids

The group of carotenoids provides the classic example of the usefulness of chromatographic analysis, as has already been mentioned on pages 13 and 26. Not only has the method been used in recent preparative work in this field, but adsorption affinity is one of the few properties in which one member of a series of carotenoids differs from another, so that chromatography has been used for purifying naturally occurring pigments that cannot be purified by other methods, such as fractional crystallisation, that are applicable to other groups of compounds.

Where a single carotenoid preponderates in some naturally occurring substance, as, for instance, lycopene in tomatoes, it can easily be obtained pure without the use of adsorption analysis. Difficulty is experienced, however, in the detection and separation of minor constituents remaining in the mother-liquors. Frequently, also, the natural pigment is a complex mixture, and the individual components have either not been prepared in the solid state, or else form mixed crystals with one another. The adsorption technique is of use not only for the purpose of isolating individual carotenoids in such instances, but also for detecting or estimating by colorimetry single polyenes that occur in plants or in animal tissues and body-fluids.

Preliminary Operations

The separation of individual pigments using one column only is not always practicable ; from complicated mixtures the total pigment is first separated into a few fractions corresponding with the various classes of carotenoids present, and each fraction is then chromatographed separately, by means of the most suitable adsorbent in each instance.

Partition between Immiscible Solvents

An excellent method of effecting a preliminary separation depends on the distribution of the components between two immiscible solvents. The solvents usually employed are light petroleum (sometimes mixed with ether) as the upper layer, and aqueous methyl alcohol (e.g. 85 to 90 per cent) as the lower layer. The distinction between " epiphasic " and " hypophasic " pigments according as, after vigorous shaking, most of the colour occurs in the upper phase or in the lower phase was known to Tswett (1). The most important carotenoids behave in a characteristic fashion and can be separated quantitatively when necessary by repeated renewal of the layer into which most of the pigment is taken.

Epiphasic carotenoids : polyene hydrocarbons $C_{40}H_{56}$.

Hypophasic carotenoids : the free polyene-alcohols and the hydroxy-ketones, e.g., capsanthin and capsorubin. The diketone rhodoxanthin is partitioned equally between the two phases. Kryptoxanthin and rubixanthin are hypophasic when the methyl alcohol is at least 95 per cent, but epiphasic if the alcohol is more dilute.

Carotenoids that are epiphasic before but hypophasic after hydrolysis : wax-pigments. The behaviour of the members of this group is reversed after treatment with methyl alcoholic-potassium hydroxide solution.

In some instances a still further separation can be carried out by the partition method.

(*a*) If an ether-light petroleum (1 : 1) solution of xanthophyll and fucoxanthin is extracted three times with 70 per cent methyl alcohol, all the fucoxanthin, with only small amounts of xanthophyll, is taken into the lower phase (Willstätter and Page).

(*b*) If 10 ml. of an ether-light petroleum (1 : 1) solution of lutein and violaxanthin are shaken four times with 2-ml. portions of 70 per cent methyl alcohol, twice as much violaxanthin as lutein passes into the lower layer (Kuhn and Winterstein 1). If now the methyl alcohol solution is shaken with 5 ml. of ether-light petroleum mixture, all the lutein, with only traces of the violaxanthin, passes into the upper layer.

It is clear that various combinations of these preparatory methods can be used prior to the adsorption analysis. For instance :

1. The total extract is subjected to a partitioning process and the free polyene alcohols are thereby obtained in the lower phase. The upper layer is separated from the lower and allowed to stand for a day in contact with concentrated methyl alcoholic potassium hydroxide solution. After washing the solution free from alkali, it is shaken with aqueous methyl alcohol. The polyene alcohols previously present in the extracts as esters pass into the lower layer and are thus separated from the hydrocarbons present. The three fractions thereby obtained are subjected to separate adsorption analysis.

2. It is sometimes sufficient simply to extract the pigments with ether, or to transfer them to ether solution and hydrolyse the esters straight away, by allowing the solution to stand for a day with 30 per cent methyl alcoholic potassium hydroxide solution in the absence of air. The solution is then diluted with water ; the ethereal layer is washed free from alkali and mixed with light petroleum and the mixture is shaken with aqueous methyl alcohol. The pigments that remain in the upper layer are chromatographed separately, as are also those that pass into the lower layer. These are recovered by extraction with ether or light petroleum after dilution with water, drying the extract so obtained with sodium sulphate, evaporation of the solvent, and dissolving the residue in a solvent suitable for adsorption.

3. It is also possible to chromatograph the mixture of pigments directly, without resorting to the preliminary separation by the partition process. If necessary, a column containing two adsorbents in the one tube can be used (see p. 133 for an example). This technique, in which no pigment

is lost, is generally used with plant extracts, but only very occasionally in working up extracts of animal origin. The fractions obtained by cutting up the column and eluting the separate zones can be subjected to partition or other methods of purification.

4. As will be seen from the examples given below, many other variations in procedure can be adopted. Thus, if only a small amount of the pigment occurs in a large quantity of material, the whole of the pigment can be concentrated on a column of strongly adsorbing material such as calcium hydroxide or alumina. A chromatogram sometimes results, but even if it does not, the pigment fraction, which now contains less colourless impurities than before, can be eluted and re-chromatographed, if necessary after some sort of preliminary treatment.

Solvents and Adsorbents. Elution

A wide choice of materials for use as solvents or adsorbents is available for separating the individual carotenoids. The three carotenes, α, β and γ, for instance, are best separated from light petroleum (b.p. 40° to 60° C.) solution with a column of calcium hydroxide (Karrer and Walker) ; but alumina with petroleum (b.p. 60° to 80° C.) or benzene-petroleum (b.p. 60° to 80° C.) mixture can also be employed, not only for the carotenes, but also for various other polyene hydrocarbons and for their esters. The amount of benzene in the mixed solvent can be varied to give more selective adsorption, but a smaller amount of pigment is thereby adsorbed with the same quantity of adsorbent. A mixture of polyene-alcohols is best separated from carbon disulphide solution on a column of calcium carbonate. The carotenoids containing oxygen can also be separated from benzene solution by a column of calcium hydroxide with the advantage that larger amounts of pigment can be accommodated on the same amount of adsorbent. The elution is more difficult, however, but can be effected with warm solvent, if necessary.

The polyenes are eluted by means of methyl alcohol or ethyl alcohol ; usually a small quantity ($\frac{1}{2}$ to 1 per cent) is added to benzene, petroleum, etc.

Brief Survey of the Adsorption Behaviour of some Carotenoids

α-, β- AND γ-CAROTENES : α-carotene has the weakest, and γ-carotene the strongest adsorption affinity. The three carotenes are adsorbed on alumina from, for instance, petroleum solution, but they are best differentiated by making use of calcium hydroxide. According to Tswett (22), carotene is the only pigment occurring in green leaves that is not adsorbed from light petroleum solution by sucrose, inulin or calcium carbonate. Calcium carbonate does not adsorb the carotenes from carbon disulphide solution.

LYCOPENE : Like the isomeric carotenes, lycopene is not adsorbed on calcium carbonate, unless the latter is of a type having exceptional adsorptive power. The adsorption affinity of lycopene is greater than that of the carotenes. Alumina, magnesia and calcium hydroxide are suitable for adsorbing lycopene from solutions in benzene or petroleum.

KRYPTOXANTHIN : Calcium hydroxide or calcium carbonate are the best adsorbents to use, with petroleum as the solvent. The column is developed with a mixture of petroleum and benzene. Kryptoxanthin, $C_{40}H_{56}O$, occupies a position in the column below that of the xanthophylls, $C_{40}H_{56}O_2$.

RUBIXANTHIN behaves similarly to kryptoxanthin and the two cannot easily be separated by chromatography.

LYCOXANTHIN and LYCOPHYLL are both adsorbed by calcium carbonate from petroleum solution. If benzene is used as solvent, calcium carbonate is ineffective ; a column of calcium hydroxide or alumina must be taken instead. Just as lycopene is more strongly adsorbed than β-carotene, so lycoxanthin (hydroxy-lycopene) is adsorbed more strongly than kryptoxanthin (hydroxy-β-carotene) and lycophyll (dihydroxy-lycopene) than zeaxanthin (dihydroxy-β-carotene).

LUTEIN (xanthophyll) is adsorbed by calcium carbonate from carbon disulphide or petroleum solution and occupies the position indicated in the table on page 28. Vitamin A and the polyene-hydrocarbons pass through the column in these circumstances.

ZEAXANTHIN behaves in a similar way to lutein. It can be

separated from β-carotene and kryptoxanthin, which have the same absorption spectra, by adsorption on calcium carbonate from solution in carbon disulphide, or a mixture (1 : 5) of benzene and petroleum. It occupies the uppermost zone of the column.

ANTHERAXANTHIN, PETALOXANTHIN and FLAVOXANTHIN (all possessing 3 hydroxyl groups) form zones in that order, reckoning from the top of the column. Anthera- and petaloxanthin are very similar to one another and have only been differentiated by the chromatographic method.

FUCOXANTHIN, VIOLAXANTHIN and TARAXANTHIN, with 6, 4 and 4 oxygen atoms respectively, can be separated chromatographically, using, for instance, a mixture of benzene and petroleum with alumina or calcium carbonate.

RHODOXANTHIN is only feebly adsorbed by calcium carbonate from petroleum solution ; it forms a zone below the polyene alcohols and this can be washed through the column into the filtrate. Rhodoxanthin forms a deep violet ring on a column of alumina from solutions in petroleum or benzene-petroleum mixture.

CAPSANTHIN and CAPSORUBIN are retained on a column of calcium carbonate from carbon disulphide or petroleum solution, and can thus be satisfactorily separated from one another. Capsanthin-ester is adsorbed (from petroleum solution) higher on the calcium carbonate column than are zeaxanthin- and lutein-esters.

The quantitative estimation of the carotenoids follows the same procedure as their isolation, the elution of the separated pigments being followed by measurement of the colour in a colorimeter or a photometer. Kuhn and Brockmann (3) worked out a systematic scheme for the estimation of the most important members of the series and used a micro-colorimeter to measure the colours of the solutions.

A solution of 14·5 mg. of pure azobenzene in 100 ml. of 96 per cent alcohol was used as a standard with a 1 cm. cell. The colour of this solution was equivalent to a petroleum (b.p. 70° to 80° C.) solution containing, in each millilitre, 0·00235 mg. of α- or β-carotene, 0·00242 mg. of kryptoxanthin, 0·00252 mg. of lutein (xanthophyll) or zeaxanthin, 0·0027 mg. of taraxanthin or violaxanthin, and 0·0046 mg. of physalien

or helenien. A solution of azobenzene ten times as strong as the above solution was equivalent to 0·0078 mg. of lycopene, 0·0095 mg. of capsanthin or 0·0098 mg. of capsorubin per millilitre.

Identification of Individual Carotenoids

The individual carotenoids that have been separated chromatographically from a mixture are best identified spectroscopically. This method is reliable even when other properties, such as the colour of the solution, colour reactions, behaviour on partitioning between solvents, and the position of the pigment on the column in relation to other pigments, do not give a clear indication of its identity. It must, however, be borne in mind that the nature of the absorption band depends not on the constitution of the molecule as a whole, but only on that of the chromophoric group. Thus, different polymers with the same chromophore have the same absorption maxima, e.g., β-carotene, kryptoxanthin and zeaxanthin (see formulæ on pp. 29–31) are spectroscopically indistinguishable, though they can readily be distinguished by partitioning between immiscible solvents.

If the positions of the observed absorption bands differ markedly from those of the carotenoid whose presence is suspected by reason of its behaviour on chromatographic analysis, then the suspicion is certainly in error. On the other hand, the identity of two polyenes is not established with certainty merely because their absorption spectra are the same; the identity can only be confirmed by a mixed chromatogram (p. 8) or, where possible, by the isolation of a crystalline compound. It is just because this latter operation was frequently omitted that the older literature is burdened with statements that are now known to be more or less incorrect.

There exists, in many carotenoids of similar structure, a simple relationship between the absorption spectrum and the adsorption affinity (p. 26; cf. Winterstein 1) which can be expressed as follows: the greater the number of conjugated double-bonds, the stronger is the adsorption affinity and the further is the absorption maximum displaced towards the longer wave-lengths of the spectrum. This is illustrated by

the behaviour of lycopene and the three carotenes on the column:

Top:	Lycopene	$C_{40}H_{56}$	13 F (11 conjugated)	548, 507 mμ (in CS_2)
	γ-Carotene	$C_{40}H_{56}$	12 F (11 conjugated)	533·5, 496 mμ
	β-Carotene	$C_{40}H_{56}$	11 F (11 conjugated)	521, 485·5 mμ
Bottom:	α-Carotene	$C_{40}H_{56}$	11 F (10 conjugated)	509, 477 mμ

With the carotenoids containing oxygen, the relationship is not always so simple as this, because the position occupied by the pigment on the column is determined to a considerable extent by the strongly active hydroxyl group. Zeaxanthin (2 OH) for instance occupies a position above that of kryptoxanthin (1 OH) although they have identical absorption maxima (519, 483 mμ in CS_2). On the other hand, the reddish-violet hydroxy-ketones capsanthin and capsorubin are adsorbed on the column above the positions occupied by the orange-yellow and yellow polyene-alcohols (cf. p. 130).

Isomerisation of the Carotenoids

The researches of Gillam and El Ridi (1, 3) and of Gillam, El Ridi and Kon into the isomerisation of the carotenoids began with the observation that the α-carotene content of butter was apparently greater with alumina as adsorbent than with magnesia. Further investigation showed, however, that the β-carotene was partially isomerised; on prolonged development of the column, there appeared underneath the main zone a second yellow zone that contained not α-carotene, but a new polyene closely resembling it, the so-called pseudo-α-carotene, $C_{40}H_{56}$. Although the two compounds have the same absorption maxima, they differ from one another in other important properties, as indicated in Table 10.

TABLE 10

COMPARISON OF α-CAROTENE AND PSEUDO-α-CAROTENE

	α-Carotene	Pseudo-α-carotene
Formula	$C_{40}H_{56}$	$C_{40}H_{56}$
Melting-point	166° (uncorr.)	187° (uncorr.)
Specific rotation (Cd light)	+ 380°	0°
Adsorption on alumina gives (partially)	"Neo-carotene"	β-carotene or similar compound

Pseudo-α-carotene is identical with natural α-carotene in chemical composition, in the amount of hydrogen it takes up, in its absorption spectrum, and in its provitamin A activity. Gillam and El Ridi (3) believed either that a *cis-trans*-isomerisation had taken place or that a double-bond had been displaced. A third possibility, however, is that pseudo-α-carotene is merely racemic α-carotene.

Gillam, El Ridi and Kon chromatographed α-carotene on a column of alumina consisting of a 1 : 1 mixture of ordinary and Brockmann alumina ; the chromatogram was developed with a mixture of light petroleum and benzene. A homogeneous zone was formed, but after elution and readsorption two zones were formed, the lower of which yielded neo-α-carotene, $C_{40}H_{56}$, of melting-point 172° C. (uncorr.). This new polyene had absorption maxima at 476 and 447 mμ in light petroleum, 487 and 457 mμ in benzene and 509 and 476·5 mμ in carbon disulphide. Its specific rotation $[\alpha]_{Cd}$ was + 220°. These isomerisations were found to be reversible.

Whereas Gillam and his co-workers attributed the isomerisation to the action of the adsorbent, Zechmeister and Tuzson (16, 17) showed that a spontaneous isomerisation of β-carotene and lycopene took place on standing or when solutions of the two substances were warmed, and that the changes have nothing to do with the actual process of chromatography. The column serves only as a convenient means of resolving the equilibrium mixture into its components (Figs. 66 and 67, p. 322), for the isomeric change can be followed readily, if somewhat less conveniently, by colorimetric or spectroscopic measurement. All carotenoids undergo an analogous change, which is catalysed by iodine. In working with polyenes, chromatography involves no risk, provided that the conditions are carefully chosen for the particular investigation in hand.

Preparation of Crystalline Pseudo-α-Carotene, $C_{40}H_{56}$, from β-Carotene, $C_{40}H_{56}$ (Gillam and El Ridi 3)

Two hundred milligrams of pure β-carotene (optically inactive, prepared from grass) were dissolved in light petroleum (b.p. 70° to 80° C.) and the solution was poured on to a column (50 × 6 cm.) of alumina made up of 1 part of Brockmann standardised alumina and 3 parts of inactive alumina. The column

was washed with light petroleum or a mixture of light petroleum and benzene. There was seldom any separation into two zones in this first chromatogram, but when the coloured band was eluted with light petroleum containing a little alcohol and the solution was washed, dried, and re-chromatographed, two zones usually formed. On prolonged development, the lower of these washed through the column into the filtrate, and was collected separately. The unchanged β-carotene retained on the column was eluted and re-chromatographed four or five times, in order to obtain the maximum degree of isomerisation. Ultimately 80 to 100 mg. of pseudo-α-carotene, dissolved in 4 litres of solvent, were obtained from 200 mg. of β-carotene, the difference being accounted for by losses due to oxidation, incomplete elution and so on. The filtrate was concentrated under reduced pressure in an atmosphere of nitrogen and filtered to remove traces of alumina. On cooling the concentrate thus obtained, crystals of the new polyene separated out. The absorption maxima were at 507 and 477 mμ in carbon disulphide and 477 and 446 mμ in petroleum, almost identical with those of α-carotene.

The authors repeated the above experiment using 100 mg. of pure β-carotene, but with their materials, and working as rapidly as possible, they obtained after six successive adsorptions a quite insignificant zone showing the pseudo-α-carotene spectrum. The absorption bands of the recovered β-carotene were, on the other hand, unaltered and sharply defined.

The spontaneous isomerisation of the polyenes is most probably a *cis-trans* rearrangement similar to that of azobenzene (p. 23).

A. Examples of the Preparation of Plant Carotenoids

(cf. also Table 11, pp. 121 to 125, and Table 12, pp. 126 and 127.)

Separation of α-, β- and γ-Carotene

According to Karrer and Walker (1) the separation of the carotenes is best conducted on a column of finely powdered commercial slaked lime from light petroleum solution; γ-carotene is retained at the top of the column and α-carotene at the bottom (see also Karrer and Schlientz). Lycopene, if present, is adsorbed above the γ-carotene zone.

TABLE 11

EXAMPLES OF THE CHROMATOGRAPHY OF POLYENES FROM HIGHER PLANTS

(Polyene-hydrocarbons are given first, then polyene-alcohols, polyene-ketones and finally pigments with less than 40 carbon atoms.)

Plant	Organ	Isolated (Detected)	Adsorbent	Solvent	Literature
Daucus carota (carrot)	Root	α-, β-Carotene	Fibrous alumina	Petroleum	Kuhn and Lederer (1)
,,	,,	,,	$Ca(OH)_2$	Light petroleum	Karrer and Walker (1)
,,	,,	α-, β-Carotene, (unknown polyene)	Fibrous alumina	,,	Karrer, Schöpp and Morf
,,	,,	α-, β- and γ-Carotene	Al_2O_3	Petroleum, benzene	Kuhn and Brockmann (7, 8)
,,	Root, leaf	α-, β-Carotene	MgO	Light petroleum	Strain (1, 2)
,,	Leaf	(Carotenes)	Norit A	Methylene chloride	Mackinney and Milner
Æsculus hippo-castanum (Horse-chestnut)	Green and autumn leaves	β-Carotene, (unknown polyenes)	$Ca(OH)_2$	Light petroleum	Karrer and Walker (2)
Urtica dioica (Stinging-nettle)	Leaf	α-, β-Carotene	,,	Petroleum	Karrer and Schlientz
Capsicum annuum (Paprika)	Fruit	α-Carotene	,,	,,	,,
Ipomœa batatas (Sweet-potato)	Tuber	β-Carotene	,,	,,	Matlack (2)
Spinacia oleracea (Spinach)	Leaf	α-, β-Carotene	,,	,,	Karrer and Schlientz
Spinacia oleracea (Spinach, cooked)	,,	β-Carotene	Al_2O_3	,,	Willstaedt and Behrnts-Jensen (1)
59 different plants	,,	α-, β-Carotene	MgO	Light petroleum	Mackinney (1)
Palm	Oil	,,	,,	,,	Strain (1, 2)
Acacia acuminata .	Wood oil	β-Carotene	Al_2O_3	Petroleum	Trikojus and Drummond

TABLE 11 (*continued*)

Plant	Organ	Isolated (Detected)	Adsorbent	Solvent	Literature
Gonocaryum pyriforme	Fruit	α-, β-, γ-Carotene, lycopene	Al_2O_3, fibrous alumina	Light petroleum, petroleum	Winterstein (2, 3)
Prunus armeniaca (Apricot)	Fruit	β-Carotene, lycopene, (γ-carotene)	Al_2O_3	Petroleum	Brockmann (1)
Tagetes erecta . .	Flowers	(Lycopene), (carotene)	$CaCO_3$	Light petroleum	Coward
Citrus grandis . . (Pink grapefruit)	Fruit	β-Carotene, lycopene	Fibrous alumina	Petroleum	Matlack
Solanum dulcamara (Woody nightshade)	Berries	Lycopene, lycophyll, lycoxanthin	$Ca(OH)_2$	Benzene	Zechmeister and Cholnoky (6)
Tropæolum, cherry, chestnut	Green and yellow leaves	(Xanthophyll), (xanthophyllester), (carotene)	$CaCO_3$	Petroleum	Kuhn and Brockmann (3)
Physalis Franchetti	Green, yellow and red sepals; older sepals; fruit-pulp; green and yellowed leaves	(Xanthophyll), (physalien), (carotene)	,,	,,	Kuhn and Brockmann (3)
Genista tridentata .	Flowers	β-Carotene, lutein	Al_2O_3	,,	Schön and Mesquita
Æsculus hippocastanum (Horse-chestnut)	Yellow leaves	β-Carotene, a xanthophyll, (unknown pigments)	$Ca(OH)_2$	Light petroleum	Karrer and Walker (2)
Citrus aurantium (Orange)	Peel and fruit-pulp	"Carotene" and "xanthophyll"			Vermast
Wheat	Grain	"Xanthophyll"	Al_2O_3	,,	Malmberg and Euler
Wheat	Germ-oil	Lutein, kryptoxanthin	,,	,,	Drummond, Singer and MacWalter
Rye	,,	(α-, β-Carotene, lutein, zeaxanthin)	MgO, Al_2O_3, sucrose	,,	Schuette and Palmer

Lycopersicum esculentum (Tomato)	Fruit	Lycopene, carotenes, zeaxanthin, lutein	Al_2O_3, $CaCO_3$	Petroleum	Kuhn and Grundmann (1)
Physalis Franchetti	Sepals, berries	Zeaxanthin, kryptoxanthin (esterified)	Al_2O_3	Benzene + petroleum	Kuhn and Grundmann (3)
Zea mays (Maize) .	Grain	Zeaxanthin, kryptoxanthin	,,	,,	Kuhn and Grundmann (5)
Rosa rugosa . .	Fruit	Rubixanthin, ("xanthophylls," lycopene, α-, β-, γ-carotene)	,,	,,	Willstaedt (5)
Rubus chamæmorus (Cloudberry)	Berry	Zeaxanthin, new xanthophyll, (β, γ-carotene, rubixanthin, lycopene ?, kryptoxanthin ?)	Al_2O_3, $Ca(OH)_2$	Benzene, petroleum	Willstaedt (9)
Vaccinium vitis idæa (Cranberry)	,,	(Lycopene, β-carotene or kryptoxanthin, zeaxanthin, xanthophyll)	,,	,,	Willstaedt (8)
Cuscuta subinclusa, *C. salina* (Dodder)		Rubixanthin, lycopene, α-, β-, γ-carotene	MgO	Light petroleum	Mackinney (2)
Leontodon autumnalis	Petals	Lutein, taraxanthin	$CaCO_3$		Kuhn and Lederer (5)
Rosa rubinosa . .	Fruit	Rubixanthin (α-, β-carotene, zeaxanthin, lutein, taraxanthin)	Al_2O_3	Benzene, petroleum	Kuhn and Grundmann (4); see also Willstaedt and With (1)
Æsculus hippocastanum	Leaf	Lutein, violaxanthin	$CaCO_3$	CS_2	Kuhn, Winterstein and Lederer
Cucurbita maxima	Fruit	α-, β-Carotene, lutein, violaxanthin	$Ca(OH)_2$, $CaCO_3$	Petroleum, CS_2	Zechmeister and Tuzson (3)

TABLE 11 (*continued*)

Plant	Organ	Isolated (Detected)	Adsorbent	Solvent	Literature
Rhubarb, plane, poplar, beech, maple, birch, weeping-willow	Green and yellow leaves	(Violaxanthin, zeaxanthin, lutein and their esters, carotene)	$CaCO_3$	Petroleum	Kuhn and Brockmann (3)
Helianthus annuus (Sunflower)	Petals	Lutein, taraxanthin, (carotene, kryptoxanthin, violaxanthin ?)	,,	CS_2	Zechmeister and Tuzson (2)
Taraxacum officinale (Dandelion)	,,	Taraxanthin, lutein, (violaxanthin ?)	,,	Petroleum	Kuhn and Lederer (2)
Ranunculus acer (Buttercup)	,,	Lutein, taraxanthin, flavoxanthin, (β-xanthophyll ?)	,,	,,	Kuhn and Brockmann (5)
Ulex europæus (Gorse)	Flowers	Carotene, lutein(iso?), viola- and taraxanthin	Al_2O_3, $CaCO_3$	Light petroleum, benzene + light petroleum	Schön
Citrus madurensis (Tangerine)	Fruit	β-Carotene, kryptoxanthin, lutein, (violaxanthin ?)	$CaCO_3$, $Ca(OH)_2$	Petroleum, CS_2	Zechmeister and Tuzson (13)
Diospyros costata .	,,	(α-), β-Carotene, lycopene, zea-, krypto- and violaxanthin	Al_2O_3, $CaCO_3$	Light petroleum, CS_2	Schön
Arbutus unedo	,,	α- and β-Carotene, (lycopene), krypto-, viola-, zeaxanthin, lutein	Al_2O_3	Light petroleum	Schön
Cucurbita pepo (Pumpkin)	Petals	Petaloxanthin, kryptoxanthin, zeaxanthin, lutein, carotene	$CaCO_3$	CS_2	Zechmeister, Béres and Ujhelyi (1, 2)
Eschscholtzia californica	,,	Eschscholtzxanthin, (flavo-, zeaxanthin, lutein, carotene)	MgO, Siliceous earth	Ethylene dichloride	Strain (7)

Thuja, etc. . . .	Leaf	(Rhodoxanthin, carotene, xanthophyll)	$CaCO_3$	Petroleum, CS_2	Lipmaa
Taxus baccata (Yew)	Aril	Rhodoxanthin, lycopene, β-carotene, zeaxanthin	Al_2O_3	Benzene + petroleum	Kuhn and Brockmann (9)
Capsicum annuum (Paprika)	Fruit-coat	Capsanthin, capsorubin, zeaxanthin, lutein, kryptoxanthin, α-, β-carotene	$CaCO_3$, $Ca(OH)_2$	CS_2, petroleum	Zechmeister and Cholnoky (3 4)
Lilium tigrinum .	Anthers	Capsanthin, antheraxanthin	$Ca(OH)_2$	Benzene	Karrer and Oswald (1)
Citrus aurantium (Orange)	Peel	Citraurin, violaxanthin, lutein, zeaxanthin, kryptoxanthin, (carotene)	$CaCO_3$, $Ca(OH)_2$	CS_2, petroleum	Zechmeister and Tuzson (14, 15) Tuzson (2)
Crocus sativus (Saffron)	Petals	Crocetin, α-, β-, γ-carotene, lycopene, zeaxanthin	Al_2O_3	Petroleum	Kuhn and Winterstein (4)
Escobedia scabrifolia and *E. linearis* (Azafranillo)	Root	Azafrin	$CaCO_3$	Benzene, petroleum	Kuhn and Deutsch
Bixa orellana (Orlean)	Seeds	" Bixol "	Al_2O_3		Bachstez and Cavallini
Gingko biloba . .	Yellow autumn leaves	(Unknown polyenes)	$Ca(OH)_2$	Light petroleum	Karrer and Walker (2)
Ulmus campestris .	Green autumn leaves	(Unknown polyenes)	,,	,,	Karrer and Walker (2)
Elodea canadensis .		Carotene, eloxanthin	Al_2O_3	Benzene + petroleum	Hey

TABLE 12

EXAMPLES OF THE CHROMATOGRAPHY OF THE POLYENES OF ALGÆ, FUNGI AND BACTERIA

Starting Material	Isolated (Detected)	Adsorbent	Solvent	Literature
Fucus vesiculosus	Fucoxanthin, zeaxanthin, β-carotene	Al_2O_3	Light petroleum	Heilbron and Phipers
Cladophora sauteri and *Nitella opaca*	β-Carotene, lutein, taraxanthin	$CaCO_3$, Al_2O_3	Benzene, petroleum	Heilbron, Parry and Phipers
Oscillatoria rubrescens . . .	Myxoxanthin, myxoxanthophyll	,,	Chloroform, petroleum	Heilbron and Lythgoe
Œdogonium	α-, β-Carotene, lutein, taraxanthin	,,	Benzene, petroleum	Heilbron, Parry and Phipers
Rhodymenia palmata . . .	β-Carotene, lutein, taraxanthin	,,	,,	Heilbron, Parry and Phipers
Euglena heliorubescens . . .	Euglenarhodone, (β-carotene, lutein, zeaxanthin, esterified)	Al_2O_3	Ether	Tischer (1)
Trentepohlia iolithus . . .	α-, β-Carotene, (lutein, zeaxanthin)	Al_2O_3, $Ca(OH)_2$	Petroleum	Tischer (2)
Hæmatococcus pluvialis . .	Hæmatoxanthin, euglenarhodone, lutein, (zeaxanthin), dipalmitate and other esters, α-, β-carotene	$CaCO_3$, $Ca(OH)_2$, Al_2O_3	Petroleum, petroleum + ether, chloroform	Tischer (3)
Aphanizomenon flos-aquæ . .	β-Carotene, aphanin, aphanicin, flavacin, aphanizophyll	Al_2O_3, $Ca(OH)_2$, Na_2SO_4	Petroleum, ether	Tischer (4)
Cantharellus cibarius, *C. lutescens*, *C. infundibiliformis*	β-Carotene, (α-carotene, lycopene, γ-carotene)	Al_2O_3, $CaCO_3$	Petroleum, benzene	Willstaedt (10)

Torula rubra	Torulene, β-carotene	Al_2O_3	Petroleum	Lederer (4)
Spirillum rubrum	Unknown polyenes, spirilloxanthin	MgO, siliceous earth	Ethylene dichloride	Van Niel and Smith
Sarcina lutea	(Sarcinin, xanthophyll)	Al_2O_3	Petroleum	Chargaff and Dieryck
Pilobolus Kleinii, Phycomyces Blakesleanus	β-Carotene	CaO	Petroleum, CS_2	Bünning (1, 2)
Sarcina lutea	(Sarcinin, a xanthophyll)	Fibrous alumina	Petroleum	Chargaff (1)
Sarcina aurantiaca	β-Carotene, zeaxanthin	Al_2O_3	,,	Chargaff (1)
Staphylococcus aureus	Zeaxanthin	,,	,,	Chargaff (1)
Mycobacterium phlei	β-, γ-Carotene, lutein	,,	,,	Chargaff (1)
Mycobacterium phlei	α-, β-Carotene, kryptoxanthin, (lutein-, zeaxanthin- and azafrin-esters)	MgO	Light petroleum	Ingraham and Steenbock
Rhodovibrio bacteria	Rhodoviolascin, rhodopin, rhodopurpurin, flavorhodin, β-carotene, lycopene, rhodovibrin	$Ca(OH)_2$	,,	Karrer and Solmssen (1, 2, 3, 6); Karrer, Solmssen and Koenig; Solmssen
Bacterium halobium	α-, β-Bacterioruberin	$CaCO_3$	CS_2	Petter (1, 2)
Thiocystis bacteria	Lycopene, rhodoviolascin	$Ca(OH)_2$	Light petroleum	Karrer and Solmssen (1, 2, 3, 6); Solmssen
Bacillus Lombardo Pellegrini	(β- and γ-Carotene)	Al_2O_3, $CaCO_3$	Petroleum, etc.	Chargaff and Lederer
Bacillus Grassbergeri	(β- and γ-Carotene, lycopene, xanthophyll)	,,	Benzene, petroleum	Chargaff and Lederer
Acid-fast bacteria from material infected with leprosy	Leprotin	Al_2O_3	Petroleum	Grundmann and Takeda

Kuhn and Brockmann (8) isolated γ-carotene from a commercial carotene preparation consisting of 15 per cent of α-, 85 per cent of β- and 0·1 per cent of γ-carotene. The crude carotene was recrystallised three times from benzene-methyl alcohol mixture and then from benzene-petroleum mixture. It was extracted twice with hot methyl alcohol and each 3-g. portion was dissolved in 300 ml. of pure benzene. The solution was diluted with 900 ml. of petroleum (b.p. 70° to 80° C.) and filtered through a column (17 × 5 cm.) of fibrous alumina (500 g.). The γ-isomer was most strongly held whilst the other two components travelled down the column, which was developed with about 1 litre of benzene-petroleum mixture (1 : 4) until a clearly differentiated red zone of γ-carotene was formed and the region of the column immediately below it was but faintly coloured. The γ-carotene zone was eluted with petroleum containing a little methyl alcohol and the solution was washed free from alcohol and re-chromatographed. The whole operation was repeated, and the final eluate was filtered and evaporated under reduced pressure, and the residue was extracted twice with hot methyl alcohol. The insoluble portion was crystallised from a little benzene-methyl alcohol mixture (1 : 1), which gave 1 mg. of crude γ-carotene per gram of original material. To effect further purification the carotene was recrystallised three times from benzene-methyl alcohol mixture (2 : 1), the crystals being extracted with methyl alcohol before each recrystallisation.

Kryptoxanthin from Maize (Kuhn and Grundmann 5)

The finely powdered, sifted meal was extracted alternately with alcohol and petroleum, each extraction being repeated several times. Water was added to the combined extracts so that the whole of the pigment passed into the petroleum. This solution was extracted with 90 per cent methyl alcohol, which removed the zeaxanthin. The petroleum solution was then hydrolysed by allowing it to stand at 35° C. for 4 hours with 5 per cent methyl alcoholic potassium hydroxide solution, and the hydrolysed solution was extracted with 90 per cent methyl alcohol, washed, dried and chromatographed on activated alumina. The column was developed with a 1 : 1 mixture of petroleum and benzene. A narrow carotene

zone washed through into the filtrate, whilst the kryptoxanthin formed an orange-yellow zone that gradually widened, but otherwise remained almost stationary. After elution of this zone with alcohol, crystalline kryptoxanthin was isolated, 700 g. of yellow Italian maize yielding 1 mg. The method can also be applied to the colorimetric estimation of kryptoxanthin, a solution of azobenzene being used as standard (p. 116).

Isolation of Taraxanthin from the Crude Crystalline Xanthophylls of Taraxacum Officinale (Kuhn and Lederer 2)

This example illustrates the use of absorption spectroscopy for identifying individual pigments when these have combined to form a single zone on the column. Forty milligrams of crude pigment were dissolved in a mixture of 25 ml. of benzene and 75 ml. of petroleum, and the solution was then drawn through a column (15 × 10 cm.) of calcium carbonate. After development of the column with 2 litres of solvent mixture, there formed a coloured zone 100 mm. thick. This was divided into five equal portions, which showed the following absorption maxima on spectroscopic examination in carbon disulphide solution :

Top :	502, 472 mμ
	502, 469 mμ
	504, 471 mμ
	507, 474 mμ
Bottom :	508, 475 mμ

The pigments from the methyl alcoholic eluates of the top two fractions were transferred to petroleum after the addition of water, and were again chromatographed. The corresponding absorption bands of the fractions from this chromatogram were :

Top :	501, 474 mμ
	501·5, 470 mμ
	501·5, 469 mμ
	501, 469 mμ
Bottom :	500·5, 468 mμ

The lutein (508, 475 mμ), therefore, was completely removed by this procedure. Fractions 2 to 5 from the second chromatogram were worked up as before and crystallised from methyl alcohol, 8 mg. of taraxanthin being thereby obtained. Its

homogeneity was established by chromatographing once more ; with the exception of the uppermost fraction (500, 473 mμ) all had the same absorption maxima (501, 468·5 mμ).

Isolation of the Pigments of Paprika.

This example demonstrates how a complicated mixture of natural substances can be studied by means of chromatographs performed in different ways, the results from one supplementing the results from another. The separation of the pigments of *Capsicum annuum*, for instance, was conducted in four different ways (Zechmeister and Cholnoky 2) :

(*a*) CHROMATOGRAPHY OF CRUDE CRYSTALLINE CAPSANTHIN yielded capsanthin, capsorubin, zeaxanthin and lutein. Without using adsorption analysis an apparently pure capsanthin is obtained, but if this is dissolved in carbon disulphide and the solution is poured on to calcium carbonate, a complex system of zones results. From the top downwards these zones are as follows, the figures at the left giving the thickness of each in mm. :

7	reddish brown	uncrystallisable, probably oxidation products
3	red	
2	deep violet	capsorubin
3	canary-yellow	unknown
28	reddish-violet	capsanthin
1	yellow	unknown
1	violet	
1	yellow	
1	deep violet	
4	lemon-yellow	
9	orange-yellow	xanthophylls
3	bright pink	unknown

(*b*) ADSORPTION ANALYSIS OF THE HYDROLYSED CRUDE EXTRACT. Paprika skins (200 g.) were percolated with 1·5 litres of light petroleum (b.p. 40° to 60° C.) and the extract, after being transferred to ether, was hydrolysed with methyl alcoholic potassium hydroxide solution. The solution was washed, dried and evaporated, and the residue was dissolved in carbon disulphide and chromatographed on 10 columns of calcium

carbonate. The chromatograms showed the following succession of zones :

5	brown
20	reddish-brown
0·2	yellow band
5	violet : capsorubin
5	canary-yellow (in carbon disulphide : 508, 476 mμ ; in ether with hydrochloric acid, blue)
20	violet : capsanthin
	five differently coloured narrow bands
5	orange-yellow : zeaxanthin
0·2	violet
10	orange-yellow : kryptoxanthin
	in the filtrate : carotene

The corresponding zones from each of the 10 chromatograms were mixed together and eluted with ether containing a little alcohol, and the extracts were evaporated and allowed to crystallise. One hundred and fifty milligrams of pure capsanthin and 18 mg. of zeaxanthin were thus obtained. The capsorubin extract, however, was chromatographed once again from carbon disulphide solution on a column of calcium carbonate ; the capsorubin now formed the main zone, with a narrow capsanthin band below it. The former was eluted and transferred to ether, and the residue was crystallised from carbon disulphide. The pigment from the kryptoxanthin zone had also to be chromatographed a second time ; use was made of a solution in petroleum and a column composed of a mixture (10 : 1) of coarse and fine calcium carbonate. The kryptoxanthin formed a light orange-yellow zone ; above it was a small zeaxanthin zone and below it a faint carotene zone. The middle zone was eluted and again chromatographed, by means of a mixture (7 : 3) of coarse and fine calcium carbonate. The edges of the zone were rejected, the remainder was eluted and the extract was transferred to benzene. The solution was washed with water, concentrated to 2 ml. and diluted with 10 ml. of methyl alcohol. Twenty milligrams of kryptoxanthin crystallised out on standing.

(c) CHROMATOGRAPHY OF NATURAL WAX-PIGMENTS WITH SUBSEQUENT HYDROLYSIS OF THE INDIVIDUAL COMPONENTS.

The fresh, fleshy starting material was dehydrated with alcohol in an atmosphere of carbon dioxide, dried and powdered and the powder (20 g.) was extracted with 500 ml. of petroleum (b.p. 70° to 80° C.) at 50° C. The extract was then poured on to 3 columns of calcium carbonate (3 parts of coarse and 1 part of fine). The chromatogram formed had the following appearance :

5 narrow bands : probably oxidation products
2 yellow
5 violet } esters of capsorubin
25 bright violet }
20 deep reddish-violet : mixture of esters of capsanthin
2 bright yellow : unknown
4 very narrow bands
filtrate : reddish yellow

The substances that are adsorbed on the column and the substances that pass into the filtrate are not necessarily the same when the esters are subjected to analysis as when the free alcohols are chromatographed, for most of the zeaxanthin ester, as well as the lutein and kryptoxanthin esters, are found in the filtrate along with the carotene.

The zones were eluted from the column by means of light petroleum containing a little alcohol. The 20-mm. band yielded a crystalline mixture of the esters of capsanthin which were converted by treatment with alkali into capsanthin itself. From this material on chromatographic adsorption only a very small amount of zeaxanthin was separated. The 5- and 25-mm. zones gave an imperfectly crystalline product that yielded crystalline capsorubin on hydrolysis. The filtrate, after treatment with alkali, was re-chromatographed from carbon disulphide solution on a column of calcium carbonate and gave the following chromatogram :

10 yellow : unknown
20 orange-yellow : zeaxanthin
30 orange-yellow : kryptoxanthin

Both these two constituents crystallised readily. The filtrate from the second chromatogram contained only polyene-hydrocarbons and after adsorption on a column of calcium

hydroxide from light petroleum solution yielded crystalline β-carotene. A trace of an unknown pigment was also obtained.

(*d*) CHROMATOGRAPHY OF HYDROLYSED AND RE-ESTERIFIED POLYENE-WAXES. It is evident, from the results obtained by chromatographic separation of the natural pigment waxes, that the same polyene-alcohol may be combined with different fatty acids, and it is sometimes advantageous in preparative work to hydrolyse these natural waxes and re-esterify the alcohols with a single acid. The synthetic esters are then chromatographed. The order in which the compounds are adsorbed on the column is independent of the nature of the fatty acid.

A solution of 400 mg. of the synthetic capric ester of crude capsanthin in 800 ml. of petroleum gave the following chromatogram, 8 columns of calcium carbonate being employed:

8 bluish-red : capsorubin-caprate
5 canary-yellow : unknown
60 brownish-red : capsanthin-caprate
10 brown : unknown
10 lemon-yellow : zeaxanthin-caprate

The main zone was re-chromatographed, but no indication of the presence of even traces of zeaxanthin was obtained. The column was eluted with benzene containing a little methyl alcohol, and the extract was washed and concentrated to a small bulk. On the addition of 4 volumes of absolute methyl alcohol capsanthin-dicaprate (240 mg.) of melting-point 109° (corr.) crystallised out.

Quantitative Estimation of the Pigments of Paprika (Cholnoky 3)

A small plug of cotton-wool was inserted into a small (5-cm.) funnel and 1 to 3 g. of powdered paprika were poured into it. The paprika was then extracted with about 200 ml. of hot petroleum (b.p. 60° to 80° C.) in 5-ml. portions, 5 minutes being allowed before each addition of solvent. When, after allowing the material to stand for 10 minutes, the filtrate obtained on the addition of fresh solvent was colourless, the extraction was regarded as complete. A column (20 × 4·5 cm.) was then prepared, the lower half of which consisted of calcium hydroxide and the upper half of calcium carbonate (1 part of fine and

4 parts of coarse). After the column had been developed with 500 to 700 ml. of petroleum, it had the following appearance (see Fig. 56, p. 318):

	several thin brown and yellow streaks	on calcium carbonate
5	red	
5	red: capsorubin-ester	
	several yellow and red streaks	
30	red: capsanthin-ester	
10	reddish-brown	
1	yellow	
5	orange-yellow: zeaxanthin-ester	on calcium hydroxide
2	lemon-yellow	
5	orange-yellow: kryptoxanthin-ester	
2	lemon-yellow	
½	orange-yellow: γ-carotene (?)	
10	orange-yellow: β-carotene	
½	ochre-yellow: α-carotene	
2	lemon-yellow	

The column was divided up, the carotenes being taken together, and each portion was eluted with petroleum containing alcohol. The alcohol was next washed out of the solution, which was then suitably diluted and the colour was measured by comparison with that of a standard solution of azobenzene (p. 116). The values obtained for capsorubin varied by 10 to 20 per cent in different experiments, but otherwise constant values were obtained.

Isolation of Lycoxanthin, $C_{40}H_{55}OH$, and Lycophyll, $C_{40}H_{54}(OH)_2$, in presence of Large Amounts of Lycopene, $C_{40}H_{56}$

Seventeen kilograms of the fresh berries of the woody nightshade (*Solanum dulcamara*) were ground in 200-g. portions with sand and the resulting paste was filtered with suction. The residue on the filter was washed twice with alcohol, and then with peroxide-free ether until the filtrate was colourless. The total quantity of ether used was 15 litres, but the faintly coloured extracts obtained towards the end of the extraction of one batch were used in the extraction of fresh material in subsequent batches. The combined extracts were washed free from alcohol and the ether was evaporated off in an atmosphere

of nitrogen under reduced pressure. The residue was dried by evaporating benzene from it once or twice, and was then treated with 3 litres of warm benzene; after the solution had been allowed to cool, it was filtered from a little resin. The solution was chromatographed on 30 columns (20 × 5·5 cm.) of calcium hydroxide and the chromatograms were developed with benzene until the region between the lycopene band and the coloured zone immediately above it was quite white. So sharp a separation between all the bands was not necessary.

3 brown: oxidation products (no spectrum)
2 deep brown: oxidation products (no spectrum)
5 red: lycophyll (503, 473, 444 mμ in hexane)
2 brown: uncrystallisable
8 red: lycoxanthin (503, 473, 444 mμ)
2 yellowish-red: unknown (494, 461, 433 mμ) rubixanthin (?)
10 yellow: not examined
100 red: lycopene (503, 473, 444 mμ)
10 yellowish-red: (493, 462, 432 mμ) γ-carotene (?)
bright yellow filtrate

The three red pigments were separately eluted with benzene-methyl alcohol mixture (3 : 1). This only presented difficulty with lycophyll, about half of which remained adsorbed on the lime. Each of the eluates was filtered, washed and dried. The crude lycophyll was chromatographed three times more, the lycoxanthin twice more, and the lycopene once more to ensure homogeneity. The final eluates were washed, dried and evaporated; the residues were dissolved in a little hot benzene, and absolute methyl alcohol was added. There were thereby obtained 920 mg. of crystalline lycopene, 125 mg. of crystalline lycoxanthin (m.p. 168° corr.) and 9 mg. of crystalline lycophyll (m.p. 179°), that is, 77, 61 and 13 per cent of the pigments estimated to be present by colorimetric assay (Zechmeister and Cholnoky 6).

Isolation of Fucoxanthin, $C_{40}H_{56}O_6$, from Brown Algæ (Heilbron and Phipers)

Fifty kilograms of *fresh*, minced *Fucus vesiculosus* were soaked for a quarter to half an hour in 50 litres of 40 per cent

acetone with frequent stirring. The mixture was filtered with suction and the solid was pressed well down to expel as much liquid as possible. The extraction was repeated with a further 50 litres of 40 per cent acetone. The extracted material was then stirred for 24 hours with methyl alcohol, and the mixture was filtered. The filtrate (40 litres) was diluted with water (10 litres) and exhaustively extracted with light petroleum (b.p. 40° to 60° C.) (Extract A). The alcoholic layer was diluted with an equal volume of water and extracted with ether. The ethereal extract was washed, dried and concentrated to about 2 litres. An equal volume of benzene was added and the mixture was distilled to remove the water present.

The residue was dissolved in 3 litres of benzene, and the solution was poured on to 15 columns (50 × 5 cm.) filled with partially inactivated alumina.[1] These were developed with a mixture (1 : 1) of benzene and light petroleum and finally washed with light petroleum (b.p. 40° to 60° C.). The chromatogram consisted of three zones :

Top :	deep olive-green : unchanged chlorophyll
	crimson : contains fucoxanthin
Bottom :	orange : decomposition products, together with a little fucoxanthin

The middle zone was eluted with methyl alcohol, and an equal volume of water was added to the eluate. A thin layer of light petroleum was poured on to the solution to reduce the risk of atmospheric oxidation. On standing, the solution slowly deposited a semi-crystalline pigment, which was filtered off, dried and recrystallised from a mixture of ether and light petroleum. Fucoxanthin (1 to 1·5 g.), identical with the material prepared by Willstätter and Page, was obtained in the form of aggregates of orange-red needles, melting-point 166° to 168° (uncorr.), having absorption maxima in carbon disulphide solution at 510, 477 and 445 mμ.

The pigment from the lowest zone of the chromatogram was transferred to light petroleum and chromatographed on fuller's earth. Two components were isolated, the one identical with fucoxanthin (2 mg.), the other, which could not be obtained in crystalline form, probably identical with "β-fucoxanthin."

[1] Use of too active adsorbent renders the separation of the zones difficult.

Fucoxanthin could not be isolated from old, dry seaweed, though zeaxanthin was isolated, 2 g. being obtained.

Extract A was concentrated in an atmosphere of nitrogen to a volume of about 3 litres, and the solution was chromatographed in 12 portions on partially inactivated alumina :

Upper :	deep olive-green : unchanged chlorophyll
	reddish-brown : pectins, fucosterol, fucoxanthin
	crimson : fucosterol, fucoxanthin
	white intermediate zone
	bluish-green : chlorophyll *a*
	white intermediate zone
Bottom :	yellow : hentriacontane, terpenes, β-carotene

Detection and Isolation of Bacterial Carotenoids

The results of investigations into the nature of bacterial carotenoids have been published by Petter (1, 2), Chargaff (1, 2), Chargaff and Lederer ; Gaffron (1, 2), van Niel and Smith ; Schneider ; Fischer and Hasenkamp (1) ; Siedel, Grundmann and Takeda ; and a summary of work in this field appears in a paper by Willstaedt (2) (cf. Table 12, p. 126).

The most comprehensive researches on bacterial carotenoids are those of Karrer and Solmssen (1, 2, 3, 6), some of which are described below (see also Karrer, Solmssen and Koenig ; Solmssen).

Lycopene (or a closely related polyene hydrocarbon) and rhodoviolascin were isolated from thiocystis bacteria, which belong to the class of red sulphur-bacteria. The chromatogram of the remaining carotenoids closely resembled that of the rhodovibrio-pigment (see below).

A culture of rhodovibrio bacteria (1,200 litres), probably containing a small amount of *Bact. chromaticum*, was used in an experiment carried out on a much larger scale. The dry bacterial mass, obtained by triturating with alcohol and filtering under suction, was extracted with carbon disulphide in a soxhlet apparatus, the red extract was evaporated under reduced pressure and the residue was treated with a mixture of petroleum and methyl alcohol. After the addition of a little water, two layers formed, the upper containing the polyene, the lower the bacterio-chlorophyll. Both layers were repeatedly extracted, and the combined petroleum extracts were then

washed and evaporated. During the partition process red crystals separated at the interface. These were filtered off and dissolved in hot benzene, and the solution was used to dissolve the residue obtained after evaporation of the petroleum solution. The liquid was transferred to ampoules, the solvent was evaporated off under reduced pressure, and the residues were sealed up until required. On extraction with light petroleum, a dark brown solution was obtained, together with crystalline rhodoviolascin, $C_{40}H_{54}(OCH_3)_2$. After two recrystallisations from benzene, the rhodoviolascin was obtained pure in a yield of 20 mg. ; it had absorption maxima at 573, 534, 496 mμ in carbon disulphide solution.

The light petroleum solution was poured on to a column of calcium hydroxide and the chromatogram was developed with the same solvent. The zones obtained were as follows (the arrows denoting further steps in the purification ; the absorption maxima were all measured in carbon disulphide solution) :

Top :	brown	↗ rhodovibrin	556, 517 mμ
	reddish-brown	→ rhodopin	547, 508 mμ
	lake	→ rhodoviolascin	573, 534 mμ
	red	→ rhodopurpurin	550, 511 mμ
	dark yellow	→ β-carotene (?)	521, 485 mμ
Bottom :	bright yellow	→ flavorhodin	502, 472 mμ

The rhodopin was eluted with a mixture of light petroleum and methyl alcohol, and the solution was washed and evaporated. The residue was crystallised first from a mixture of petroleum with a little benzene and then from absolute alcohol. The rhodopin was further purified by re-chromatographing on calcium hydroxide, the column being developed with a mixture (1 : 1 or 1 : 2) of light petroleum and benzene. The rhodopin zone of this second chromatogram was arbitrarily divided into three equal portions ; the top fraction yielded rhodovibrin, melting-point 168° C., and the lowest rhodopin. By repetition of this treatment, Karrer, Solmssen and Koenig ultimately obtained pure rhodopin, melting-point 171° C.

Rhodopurpurin was obtained in the form of needles melting at 161° to 162° C. by elution of the appropriate zone, transference to light petroleum and evaporation of the solution.

B. Examples of the Isolation of Carotenoids from Animal Sources

(Cf. also Tables 13, p. 140, and 14, p. 142, as well as Zechmeister 6.)

Estimation of Lipochromes in Human Liver

The fresh, minced tissue, dehydrated by repeated treatment with alcohol, was allowed to stand overnight in contact with ether, which extracted most of the carotenoids, the rest being readily removed on extraction with a further quantity of fresh ether. The solution was hydrolysed by standing with concentrated methyl alcoholic potassium hydroxide solution for 16 to 20 hours in an atmosphere of nitrogen. The ethereal solution was washed, dried and evaporated, and the residue was dissolved in petroleum. This solution was next shaken with aqueous methyl alcohol, the lower layer was diluted with water and the hypophasic pigments were then transferred to petroleum. This solution and that containing the epiphasic pigments were separately chromatographed on columns of calcium hydroxide, and the individual zones were eluted with ether containing a little methyl alcohol. The residues obtained on evaporation of the solvent were dissolved in petroleum, and the colours of the resulting solutions were compared with that of a standard solution of azobenzene (p. 116).

The epiphasic pigments gave two main zones, the upper containing lycopene, the lower carotene. The hypophasic pigments also gave two main zones, the upper due to xanthophyll, the lower to an unknown decomposition product. The spectroscopic control of the process of separation was facilitated by re-chromatographing the fractions, the bands being then more sharply defined. Each kilogram of fresh liver was by this method found to contain 1·6 mg. of carotene, 0·5 mg. of lycopene, 0·5 mg. of xanthophyll and 0·05 mg. of an unknown polyene (Zechmeister and Tuzson 9).

Willstaedt and Lindquist have recently found in human liver, carotene, lycopene, zeaxanthin, xanthophyll, and possibly also violaxanthin, by a similar method, using columns of alumina. They also mention unknown coloured constituents, polyene degradation products.

TABLE 13

EXAMPLES OF THE CHROMATOGRAPHY OF POLYENES FROM THE HUMAN ORGANISM

Starting Material	Isolated (Detected)	Adsorbent	Solvent	Literature
Blood-serum	(Lycopene, carotene, xanthophylls)	$Ca(OH)_2$	Petroleum	Dániel and Scheff; Dániel and Béres
,,	(Carotenes, xanthophylls)	Al_2O_3	,,	Süllmann, Szécsényi-Nagy and Verzár
,,	(Carotene, lycopene, zeaxanthin, unknown xanthophylls)	,,	,,	Willstaedt and Lindquist
,,	(Carotenes, kryptoxanthin)	,,	Light petroleum	Van Veen and Lanzing
,,	(β-Carotene, xanthophylls)	,,	Petroleum + benzene	Willstaedt and With (1)
Milk	,,	,,	,,	Willstaedt and With (2)
Liver	(Carotene), (lycopene), (xanthophyll)	$Ca(OH)_2$	Petroleum	Zechmeister and Tuzson (9)
,,	(Carotene, lycopene, zeaxanthin, lutein, violaxanthin)	Al_2O_3	,,	Willstaedt and Lindquist
Depot-fat	β-Carotene (lycopene, capsanthin, xanthophyll)	$Ca(OH)_2$	,,	Zechmeister and Tuzson (8)
Placenta	(Xanthophylls, xanthophyll ester, carotene)	$CaCO_3$	,,	Kuhn and Brockmann (3)
Fæces	(Carotene, xanthophyll)	Al_2O_3	,,	Van Eekelen and Pannevis

Chromatography of the Lipochromes of Blood-serum

Carotenoids are present in the blood sera of various animals; after liberation from their complexes with protein they can be separated and estimated by adsorption analysis. The earliest and most important investigations into this matter are those of Palmer and Eckles and are concerned with the lipochromes in the blood-stream of the cow; Gillam and El Ridi (1, 2) established the presence there of lutein and kryptoxanthin, together with vitamin A and much carotene. Horse serum was shown by chromatography to contain predominantly carotene as well as coloured degradation products of polyenes from foodstuffs (Zechmeister and Tuzson 10).

SEPARATION OF THE CAROTENOIDS OF HUMAN BLOOD-SERUM. In the literature, the amount of ether-soluble pigments in the serum is frequently regarded as equivalent to the carotene content, an assumption that has been shown by recent work to be untenable (Dániel and Scheff; Dániel and Béres). The total pigments must at the very least be partitioned between immiscible solvents; if appreciable amounts of the highly coloured lycopene are present, the epiphasic fraction must also be chromatographed, otherwise high results are obtained and lead to an exaggerated estimate of the amount of provitamin A present. Three litres of human blood-serum were precipitated with an equal volume of alcohol and the precipitate was centrifuged off and extracted exhaustively with peroxide-free ether. The extract was hydrolysed by treating it with methyl alcoholic potassium hydroxide solution and allowing the mixture to stand for two days in an atmosphere of nitrogen; the pigments were transferred to petroleum and were then partitioned between this solvent and 90 per cent methyl alcohol. The epiphasic pigments were freed from cholesterol and chromatographed on calcium hydroxide. The amount of each pigment present was estimated micro-colorimetrically.

Top:	yellowish-brown: unknown (0·3 mg., calculated as carotene)
	rose-red: lycopene (0·15 mg.)
	light yellow: unknown (0·08 mg.)
Bottom:	orange yellow: β- (and α-) carotene (0·28 mg.)

The hypophasic portion contained 0·84 mg. of "lutein."

TABLE 14

Examples of the Chromatography of Animal Polyenes

Animal	Organ, etc.	Isolated (Detected)	Adsorbent	Solvent	Literature
Cow	Blood-serum	(Carotene, xanthophyll)	$CaCO_3$	CS_2	Palmer and Eckles
,,	Fat	Carotene	$Ca(OH)_2$	Petroleum	Zechmeister and Tuzson (1)
,,	Corpus luteum	(α-, β-Carotene)	$CaCO_3$	,,	Karrer and Schlientz
,,	Corpus rubrum	β-Carotene	Fibrous Alumina + Al_2O_3		Kuhn and Brockmann (3)
,,	Milk	(Lycopene, carotene)	Al_2O_3	Petroleum + benzene	Van Wijngaarden, Willstaedt and With (1, 2)
,,	Butter	Xanthophyll, (lutein), zeaxanthin, carotene, (vitamin A)	$CaCO_3$, Al_2O_3	Light petroleum	Karrer and Schöpp (1)
,,	,,	(Carotene, lycopene, xanthophyll, kryptoxanthin)	Al_2O_3	Petroleum	Gillam and Heilbron (1)
,,	,,	β-Carotene	MgO + Hyflo Supercel	,,	Gillam and El Ridi (4)
,,	,,	(Carotene, xanthophyll)	$CaCO_3$	CS_2	Palmer and Eckles
,,	Dung	,,	,,	,,	,,
Cattle	Retina	(β-Carotene)	Al_2O_3	Petroleum	Brunner, Baroni and Kleinau
Ox	Blood-serum	(β-, α-Carotene)	,,	Petroleum + benzene	Willstaedt and With
Pig	Retina	(β-Carotene)	,,	Petroleum	Brunner, Baroni and Kleinau

Horse	Blood-serum, liver, adipose tissue, kidneys, adrenals, lungs, spleen	(Carotene, degradation products)	$Ca(OH)_2$	”	Zechmeister and Tuzson (10)
”	Fat	(α-), β-Carotene	”	”	Zechmeister and Tuzson (4)
”	Dung	”	$CaCO_3$, $Ca(OH)_2$	Petroleum, CS_2	Zechmeister and Tuzson (5)
Pig	Liver	(Negative result)	$Ca(OH)_2$	Petroleum	Zechmeister and Tuzson (12)
Rat	Dung	(Physalien, zeaxanthin)	$CaCO_3$	”	Kuhn and Brockmann (3)
Whale	Oil	Astacene	Al_2O_3, fuller's earth	”	Burkhardt, Heilbron, Jackson, Parry and Lovern
Balænoptera musculus (Blue whale)	”	(Carotenoid)	Talc, $CaCO_3$	”	Schmidt-Nielsen, Sörensen and Trumpy (2)
Hen	Fat	(Carotene, violaxanthin), xanthophyll	$CaCO_3$	CS_2	Zechmeister and Tuzson (4)
”	Egg-yolk	Lutein, zeaxanthin	”	”	Kuhn, Winterstein and Lederer
”	”	(Lutein, esterified lutein, carotene)	”	Petroleum	Kuhn and Brockmann (3)
”	”	(Carotene, kryptoxanthin, xanthophylls, vitamin A)	$CaCO_3$, Al_2O_3	”	Gillam and Heilbron (2)
”	”	(Xanthophylls)	Al_2O_3	”	Willstaedt and With (1)
”	”	Lutein, zeaxanthin	$MgCO_3$		Euler and Gard
”	Retina	Polyene-hydrocarbons, xanthophyll, astacene	CaO, $CaCO_3$, Al_2O_3	Petroleum	Wald and Zussman
Hen, goose, duck	Tarsal skin	(Lutein ester, “canary-xanthophyll”)	$CaCO_3$	”	Brockmann and Völker

TABLE 14 (*continued*)

Animal	Organ, etc.	Isolated (Detected)	Adsorbent	Solvent	Literature
Goose	Beak-epidermis	(Lutein ester, "canary-xanthophyll," carotene	$CaCO_2$	Petroleum	Brockmann and Völker
Pigeon	Tarsal skin	(Zeaxanthin, lutein, red decomposition product)	,,	,,	,,
Numerous kinds of birds	Plumage	(Lutein, zeaxanthin, "canary-xanthophyll," picofulvin, decomposition products)	,,	,,	Brockmann and Völker ; Völker
Rana esculenta (Edible frog)	Liver, skin, ovaries, fat	α-, β-Carotene, lutein, zeaxanthin	$Ca(OH)_2$	,,	Zechmeister and Tuzson (11)
,,	,,	(β-Carotene, lutein)	$CaCO_3$		Brunner and Stein
Rana catesbiana (Bull frog)	Retina	Xanthophyll	$CaCO_3$, Al_2O_3	Petroleum	Wald
Regalecus glesne . .	Liver-oil	Carotenoid	$CaCO_3$	,,	Schmidt-Nielsen, Sörensen and Trumpy (1)
Hippoglossus hippoglossus (Halibut)	Roe	(Xanthophyll, zeaxanthin)	,,	CS_2	Euler, Gard and Hellström
Lophius piscatorius (Sea-devil)	Liver	(Unknown carotenoids)	,,	,,	Sörensen (2)
,,	Liver-oil	(Astacene, taraxanthin ?)	Al_2O_3	Petroleum	Burkhardt, Heilbron, Jackson, Parry and Lovern
Nephrops norvegicus (Shrimp)	Whole body	Astacene	,,	,,	,,
Orthagoriscus mola .	Liver	(α- and β-Carotene, unknown polyenes)	,,	,,	Sörensen (1)
Salmo salar (Salmon)	Muscle	Salmic acid	,,	,,	Emmerie, Van Eekelen, Josephy and Wolff
Hypsypops rubicunda (a marine goldfish)		Xanthophyll	$CaCO_3$	,,	Fox (1)

Botryllus Schlosseri .		Pectenoxanthin, capsorubin, capsanthin, lycopene, β-carotene	,,	Petroleum + benzene	Lederer (7)
Dendrodoa grossularia		Astacene ester	,,	,,	,,
Halocynthia papillosa (*Cynthia pap.*)		Astacene ester, " cynthiaxanthin "	,,	,,	,,
Maja squinado (Sea spider)	Eggs	(" Vitellorubin," carotene)	,,	Petroleum	Dhéré and Vegezzi, cf. Vegezzi
Mytilus californianus (Californian mussel)	Fæces	(Carotene, xanthophyll)	,,	Light petroleum	Fox (2)
Pecten maximus (Scallop)	Sexual organ	Pectenoxanthin	,,		Lederer (8)
Cardium tuberculatum (Cockle)		(Unknown polyenes)	$Ca(OH)_2$	Benzene	Karrer and Solmssen (5)
Echinus esculentus (Sea-urchin)	Sexual glands	Echinenone	$Ca(OH)_2$, Al_2O_3	Petroleum	Lederer (10) ; Lederer and Moore
,,	Whole animal	Pentaxanthin	Al_2O_3, $CaCO_3$	Benzene	Lederer (8)
Tealia felina . . .		Actinio-erythrin ?-ester, (carotene)	,,	,,	Heilbron, Jackson and Jones
Actinoloba dianthus (Sea pink)		New polyene acid	$Ca(OH)_2$	Light petroleum	,,
Anemonia sulcata (Sea rose)		Sulcatoxanthin, (carotene)	Al_2O_3	Benzene	,,
Actinia equina . .		Actinio-erythrin	$CaCO_3$	Petroleum	Fabre and Lederer ; Lederer (5)
,,		,,	Al_2O_3	Light petroleum	Heilbron, Jackson and Jones
Axinella cristagalli		Astacene, (unknown pigments)	$CaCO_3$	Benzene	Karrer and Solmssen (5)
Coccinella septempunctata	Wing covering	(Lycopene, carotenes)	Al_2O_3	Petroleum	Lederer (6)
Pyrrhocoris apterus .	,,	(Lycopene)	,,	,,	,,
Œdipoda miniata .	,,	(β-Carotene)	CaO	,,	,,

It follows from these results, therefore, that for clinical purposes a reliable estimate of the carotene-content of blood-serum can only be obtained when sufficient is available for an adsorption analysis.

Willstaedt and Lindquist developed a method that required only 140 to 250 ml. of blood-serum. They evaporated the ethereal extract in a current of nitrogen, dissolved the sterol-containing residue in light petroleum, and extracted the solution with 90 per cent methyl alcohol. Both fractions were chromatographed on columns of alumina, which were developed with a mixture of petroleum and benzene. The appropriate zones were eluted and estimated micro-colorimetrically as described above.

The epiphasic fraction contained lycopene and carotene, in an amount agreeing with that found by Dániel *et al.* Between the two main zones of the chromatogram were two bands, coloured pink and brownish-yellow respectively, caused by two unknown degradation products possessing absorption maxima (in light petroleum) at 498, 468 and 442 mμ and at 478 and 459 mμ respectively. Complete separation was only achieved after repeated chromatography and development of the columns with large amounts of a mixture (4 : 1) of benzene and petroleum. Zeaxanthin and xanthophyll were both ordinarily found in the hypophasic portion. For the chromatographic estimation of serum-carotenoids in the presence of bilirubin, see Bile-pigments (p. 106 ; Süllmann, Szécsényi-Nagy and Verzár).

Isolation of Carotene from Horse-fat

Two kilograms of the minced material were added to a solution of 500 g. of potassium hydroxide in 3 litres of 96 per cent alcohol and warmed for 15 minutes at 50°. To the solution, cooled to 25°, 8 litres of ether were added and water was then added in small amounts at a time until two layers separated. The lower layer was tapped off and extracted by gentle shaking with ether. The combined ethereal extracts were allowed to stand for a day over concentrated methyl alcoholic potassium hydroxide solution, water was then added, and the ethereal solution was washed until free from alkali, dried over anhydrous sodium sulphate and evaporated. The residue was partitioned

between light petroleum (b.p. 30° to 60° C.) and 90 per cent methyl alcohol; most of the pigments were epiphasic.

The washed and dried light petroleum solution was chromatographed on a column (18 × 4 cm.) of calcium hydroxide. Colourless components accumulated in the upper half of the column, whilst the pigment gradually moved down the column as washing proceeded. When the coloured zone was half-way down the column, it separated into two bands, the upper orange-yellow containing β-carotene, 10 mm. thick, the lower lemon-yellow containing α-carotene, 5 mm. thick. The β-carotene zone was eluted with ether containing methyl alcohol, and the eluates were washed with water, dried and evaporated. The residue yielded from 0·3 ml. of benzene and a few millilitres of methyl alcohol, 3 mg. of crystalline β-carotene, corresponding with 25 per cent of the quantity estimated by colorimetric measurement to be present in the fat (Zechmeister and Tuzson 4).

Carotene and Xanthophyll (Lutein) from Horse Fæces

Two kilograms of the fresh fæces were allowed to stand for 3 hours with 3 litres of 5 per cent alcoholic potassium hydroxide solution, the mixture was filtered, and the residue was washed with alcohol and then thoroughly extracted with ether (3 litres). The alkaline filtrate was also extracted with ether, the combined ethereal extracts were dried with sodium sulphate and evaporated; the residue was partitioned several times between (*a*) petroleum and (*b*) 90 per cent methyl alcohol.

(*a*) The petroleum solution was twice chromatographed on calcium hydroxide. Apart from a small amount of α-carotene, the chromatogram consisted of a bright yellow zone of β-carotene. This was eluted with ether containing a little methyl alcohol, and the eluate was evaporated. To the residue was added a little benzene and then methyl alcohol and a small amount of absolute alcohol. Sterols that first crystallised out were filtered off, and from the filtrate crystals of β-carotene (8·6 mg.) separated out on standing in the ice-chest.

(*b*) The polyene-alcohols were transferred from methyl alcohol to carbon disulphide and the solution was poured on to a column of calcium carbonate. The main zone was of a deep orange-yellow colour and this was eluted and re-adsorbed.

The main zone of the new chromatogram was eluted as above; the dry residue from the eluate was dissolved in 1 ml. of chloroform and several volumes of light petroleum were added. On standing overnight at 0° C., crystalline xanthophyll (32 mg.) separated out. This was dissolved in hot methyl alcohol, filtered at 20° C. from colourless impurities, and purified by re-chromatographing on a column of calcium carbonate. The pigment was eluted and crystallised as before from chloroform-light petroleum mixture. The yield of pure xanthophyll was 50 to 60 per cent of that estimated colorimetrically to be present (Zechmeister and Tuzson 5).

Isolation of Actinio-erythrin from Actinia Equina (Fabre and Lederer)

This investigation provides an example of the isolation of a polyene-ester from animal sources. The anemones (red variety) were minced and extracted several times with acetone, giving a reddish-violet solution, from which the pigment was transferred to light petroleum after addition of water. The petroleum solution was washed and then shaken with 90 per cent methyl alcohol, but the lower layer remained colourless. The upper layer was therefore washed free from alcohol with water; then, after standing in the ice-chest for some time, it was decanted from the droplets of water that had formed and chromatographed on a column of calcium carbonate. The reddish-violet zone that formed moved slowly down the column and ultimately separated into three bands. The chief of these was eluted with light petroleum containing 1 per cent of methyl alcohol, the solution was filtered and evaporated under reduced pressure, and the residue was dissolved in absolute alcohol. Water was added drop by drop until the solution showed a slight turbidity, when it was placed in the ice-chest. On standing, it deposited greyish-violet needles of actinio-erythrin; 1·5 mg. were obtained from 30 animals, about 30 per cent of the estimated pigment content.

Carotenoids of Deep-sea Mud and Sea-water (probably partially of vegetable origin: Fox 3)

About 200 g. of fine mud from a depth of 2,000 m. were extracted with methyl alcohol and the pigment was partitioned

between methyl alcohol and light petroleum. The saponified epiphasic fraction gave chiefly α- and β-carotenes on a column of calcium hydroxide, whilst the hypophasic fraction gave a polyene apparently identical with lutein when chromatographed on calcium carbonate.

Four thousand litres of sea-water were filtered through cotton-wool and the residue so obtained was used for adsorption experiments. Its behaviour on a column of calcium carbonate indicated the presence of pigment-waxes.

For the separation of carotenoids and vitamin A, see page 248.

C. Examples of the Isolation of Synthetic Derivatives of the Carotenoids

(Cf. also Table 15, pp. 150 to 152.)

Separation of Carotene and Isocarotene

Isocarotene, which, according to Karrer, is dehydro-β-carotene, $C_{40}H_{54}$ (Zechmeister 1), prepared, for example, by iodinating natural carotene and subsequently regenerating with sodium thiosulphate, was separated (Kuhn and Lederer 4) from β-carotene on a column made of 1 part of fibrous alumina and 2 to 4 parts of alumina (Merck), using petroleum for developing the chromatogram. Isocarotene was more strongly adsorbed than carotene, its adsorbability being about the same as that of lycopene.

Preparation of Neo-lycopene, and its Separation from Lycopene

If a freshly prepared solution of pure lycopene in benzene is poured on to a column of calcium hydroxide, a chromatogram consisting of a broad uniformly pink band is formed, though occasionally a second smaller band may make its appearance, owing to traces of a contaminating polyene. When, however, 35 mg. of lycopene in 50 ml. of benzene were boiled under reflux for half an hour, there was formed an appreciable quantity of neo-lycopene. The solution was cooled, diluted with 25 ml. of petroleum, and chromatographed on calcium hydroxide. The chromatogram was developed with a mixture (1 : 3) of petroleum and benzene until the yellow-brown zone

TABLE 15

Examples of the Chromatography of Transformation and Degradation Products of Carotenoids

Starting-Material (Addition)	Isolated (Detected)	Adsorbent	Solvent	Literature
β-Carotene, isomerisation	Pseudo-α-carotene	Al_2O_3	Light petroleum	Gillam and El Ridi (1, 3)
β-Carotene + iodine + thiosulphate	β-Carotene, isocarotene, (dehydro-β-carotene)	Fibrous alumina + Al_2O_3	Petroleum	Kuhn and Lederer (4)
β-Carotene + perbenzoic acid	Carotene oxide	Fibrous alumina	Light petroleum	Euler, Karrer and Walker
β-Carotene + chromic acid	Semi-β-carotenone, β-carotenone, β-carotene	Al_2O_3	Benzene + petroleum	Kuhn and Brockmann (10)
”	β-Hydroxy-carotene, β-carotenone	Al_2O_3 $CaCO_3$	Petroleum, light petroleum	Kuhn and Brockmann (6)
”	β-Carotenone, β-hydroxy-carotene, semi-β-carotenone, β-hydroxy-semi-carotenone, β-carotenone-aldehyde	Al_2O_3	Benzene + petroleum	Kuhn and Brockmann (14)
”	Neo-β-hydroxy-carotene, semi-β-carotenone	”	Benzene	Karrer and Solmssen (1)
β-Carotene + permanganate	β-Carotenal	$Ca(OH)_2$	Petroleum	Karrer and Solmssen (7)
”	β-Apo-4-carotenal	Al_2O_3	Light petroleum + benzene	Karrer, Solmssen and Gugelmann
β-Apo-2-carotenal, reduced	β-Apo-2-carotenol	$Ca(OH)_2$	”	Euler, Karrer and Solmssen
α-Carotene, isomerisation	Neo-α-carotene	Al_2O_3	Petroleum	Gillam, El Ridi and Kon
α-Carotene + permanganate	α-Apo-2-carotenal	$Ca(OH)_2$	Light petroleum	Euler, Karrer and Solmssen
α-Carotene + chromic acid	α-Hydroxy-carotene, α-carotone			Karrer, Salomon and Schöpp
”	α-Hydroxy-carotene, α-carotone, semi-α-carotenone	Al_2O_3	Benzene + petroleum	Karrer, Euler and Solmssen
β-Hydroxy-carotene + chromic acid	Azafrinone-aldehyde	$CaCO_3$	”	Kuhn and Brockmann (13)

β-Carotenone + chromic acid	β-Carotenone-aldehyde	$CaCO_3$ + talc, Al_2O_3	,,	Kuhn and Brockmann (14)
β-Carotenone-aldehyde . . .	Oxime	$CaCO_3$	Benzene	,,
β-Carotenone + methyl alcoholic KOH	Bisanhydro-β-carotenone	Al_2O_3	Benzene + petroleum	,,
Anhydro-β-carotenone . . .	Oxime	,,	Benzene	,,
Lycopene, isomerisation . .	(Neo-lycopene)	$Ca(OH)_2$	Benzene + petroleum	Zechmeister and Tuzson (16, 17)
Lycopene + chromic acid . .	Bixin-dialdehyde	$CaCO_3$	Petroleum	Kuhn and Grundmann (1)
,, . .	Lycopenal	Fibrous alumina + Al_2O_3	,,	Kuhn and Grundmann (2)
Lycopenal	Oxime	$CaCO_3$	,,	,,
Kryptoxanthin, isomerisation .	(Neo-kryptoxanthin)	$Ca(OH)_2$	,,	Zechmeister and Tuzson (17)
Physalien + chromic acid . .	Physalienone			Karrer, Salomon and Schöpp
Xanthophyll acetate + permanganate	α-Citraurin	,,	Light petroleum + benzene	Karrer, Koenig and Solmssen
Zeaxanthin acetate + permanganate	β-Citraurin	,,	,,	Karrer, Ruegger and Solmssen
Dihydro-rhodoxanthin + Al isopropylate	Zeaxanthin	,,	Benzene	Karrer and Solmssen (4)
Capsanthin ester	(Purification)	$CaCO_3$	Petroleum	Zechmeister and Cholnoky (2)
Capsanthin + Al isopropylate	Capsanthol	$Ca(OH)_2$	Benzene	Karrer and Hübner
Capsanthin diacetate + chromic acid	4-Hydroxy-β-carotenone aldehyde oxime	$CaCO_3$	,,	Zechmeister and Cholnoky (4)
,,	Capsyl aldehyde oxime	,,	Benzene + petroleum	,,
,,	Capsanthinone, 4-hydroxy-β-carotenone aldehyde, capsyl aldehyde, capsanthylal	,,	,,	,,
Capsanthylal	Oxime	,,	Benzene	,,
Capsanthin + alcoholic KOH .	Citraurin	,,	Benzene + petroleum	Zechmeister and Cholnoky (7)

TABLE 15 (*continued*)

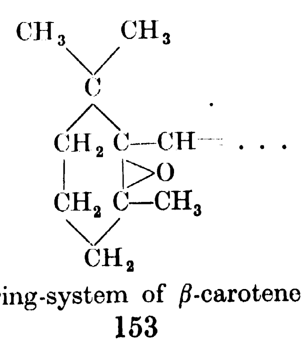
Starting-Material (Addition)	Isolated (Detected)	Adsorbent	Solvent	Literature
Capsanthinone + methyl alcoholic KOH	Anhydro-capsanthinone, (unknown pigments)	$CaCO_3$	Benzene + petroleum	Zechmeister and Cholnoky (4)
Capsorubin diacetate . . .	(Purification)	,,	Petroleum	Zechmeister and Cholnoky (3)
Bixin methyl ester (labile) + permanganate	Apo-3-norbixinal methyl ester	$Ca(OH)_2$	Benzene + light petroleum	Karrer and Solmssen (8)
Bixin methyl ester (stable) + permanganate	Apo-3-norbixinal methyl ester, apo-2-norbixinal methyl ester	,,	,,	Karrer and Solmssen (8)
Bixin (labile) + permanganate	Apo-1-norbixinal methyl ester	,,	,,	,,
Bixin (stable) + permanganate	,,	,,	,,	,,
Crocetin dimethyl ester . .	(Purification)	Al_2O_3	Petroleum	Kuhn and Winterstein (3)
Crocetin dimethyl ester, thermal decomposition	Octatetraene-dicarboxylic acid dimethyl ester, tricyclocrocetin ester, 2 : 6-dimethylnaphthalene, etc.	,,	,,	,,
Azafrin	Apo-1-azafrinal	$Ca(OH)_2$	Benzene + light petroleum	Karrer, Obst and Solmssen
Azafrin methyl ester + chromic acid	Azafrinone methyl ester	Al_2O_3	Petroleum	Kuhn and Deutsch
,,	Azafrinone methyl ester, azafrinal-I-methyl ester, azafrinal-II-methyl ester	,,	Benzene	Kuhn and Brockmann (14)
Azafrinone	Amide	$CaCO_3$	,,	,,
Anhydro-azafrinonamide . .	(Purification)	,,	,,	,,
Anhydro-azafrinone methyl ester	Oxime	Al_2O_3	,,	,,
Azafrinal-I-methyl ester . .	,,	,,	,,	,,
Azafrinal-I-methyl ester oxime	Nitrile	,,	,,	,,
Azafrinal-II-methyl ester oxime	,,	,,	,,	,,

of neo-lycopene formed below the lycopene zone was separated from it by a completely white region. The quantity of the new pigment formed was 50 to 55 per cent of the weight of starting material. It gave the following absorption maxima: 536, 498, 466 mμ in carbon disulphide, 512, 479, 450 mμ in benzene, and 499·5, 468, 439 mμ in petroleum. A solution of 0·0032 mg. in 1 ml. of petroleum had the same colour-intensity as a solution of 14·5 mg. of azobenzene in 100 ml. of alcohol. If the neo-lycopene zone is eluted with petroleum containing alcohol, and the solution is washed, dried, allowed to stand for some time and then poured on to a column, a lycopene band is formed above that due to neo-lycopene (Zechmeister and Tuzson 16, 17, Figs. 66 and 67, p. 322).

β-Carotene Monoxide (Euler, Karrer and Walker)

A solution of 0·536 g. of carotene in 100 ml. of chloroform was treated with 5 ml. of perbenzoic acid solution (= 0·166 g. of oxygen) and allowed to stand for 36 hours in the dark. The brownish-red solution was then shaken twice with very dilute sodium bicarbonate solution and dried by pouring it through a folded filter. The filtrate was evaporated, the residue was dissolved in benzene, and methyl alcohol was added until a faint turbidity was formed. After standing for 12 hours at 0° C., crystals separated out; these were filtered off, washed with methyl alcohol, and dissolved in 50 ml. of light petroleum. The solution was chromatographed on fibrous alumina, giving the following chromatogram:

Top:	10 reddish-yellow, not crystallised
	45 yellow, carotene monoxide
Bottom:	25 red (isocarotene ? 542, 503, 472 mμ in carbon disulphide)
	Filtrate reddish-yellow



Probable ring-system of β-carotene-monoxide

The 45-mm. zone was eluted with 90 per cent methyl alcohol and the pigment was transferred to light petroleum; the residue remaining on evaporation of the solvent was crystallised, first from benzene-methyl alcohol mixture, and then several times from light petroleum. A yield of 30 to 70 mg. of carotene oxide, $C_{40}H_{56}O$, was obtained; the new compound had a melting-point of 160° to 161° C. and absorption maxima in carbon disulphide at 486, 456, (427) mμ.

Preparation of β-Hydroxy-Carotene, β-Carotenone and Semi-β-Carotenone from β-Carotene (Kuhn and Brockmann 6, 10, 13, 14; formulæ, Table Y, p. 32)

Three grams of carotene were divided into 50-mg. portions and each portion was dissolved in 15 ml. of benzene; each solution was diluted with 25 ml. of glacial acetic acid, and 3 ml. of 0·1*N* chromic acid were added drop by drop with vigorous stirring. After the addition of an equal volume of petroleum (b.p. 70° to 80° C.), the solution was poured into water and the upper layer was washed, dried, and poured on to Brockmann alumina. The following chromatogram (60 columns) was obtained on developing with benzene:

Top:	brownish-red (472, 443, 423 mμ in petroleum)
	reddish-violet (502, 470, 446 mμ) β-carotenone
	yellowish-red (478, 448, 420 mμ) β-hydroxy-carotene
Bottom:	reddish-violet (502, 470, 446 mμ) semi-β-carotenone, yellow (453, 425 mμ)
	Filtrate: unchanged carotene

Each of the zones was separately eluted with benzene containing methyl alcohol, and the eluates were washed, dried and evaporated. The β-carotenone (110 mg.) was crystallised from benzene and petroleum, the β-hydroxy-carotene (285 mg.) from benzene and methyl alcohol, and the semi-β-carotenone after a further chromatographic separation (240 mg.) from methyl alcohol. Unchanged β-carotene (500 mg.) was recovered from the filtrate.

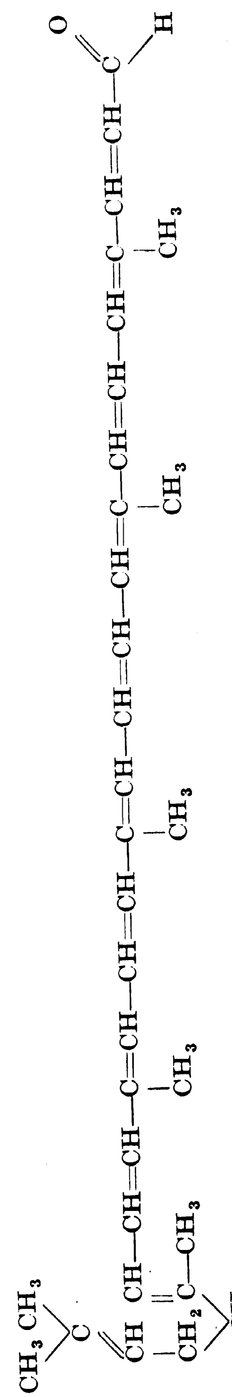
Lycopenal from Lycopene (Kuhn and Grundmann 2)

A solution of 100 mg. of lycopene in 70 ml. of pure benzene was treated with 100 ml. of glacial acetic acid (distilled over potassium permanganate) and then, drop by drop with vigorous

stirring, with 11·2 ml. of 0·1N chromic acid; 200 ml. of petroleum (b.p. 70° to 80° C.) and 500 ml. of doubly distilled water were added; the upper layer was washed until free from acid, and then shaken ten times with 20 ml. of 90 per cent methyl alcohol to remove by-products formed during the oxidation. After being washed, the petroleum solution was chromatographed on a mixture of fibrous alumina (1 part) and alumina (4 parts). The lycopenal, $C_{32}H_{42}O$, remained on the column after development with petroleum, and was eluted with chloroform containing 10 per cent of absolute alcohol. The eluate was concentrated to about 1 ml. and, after the addition of 10 ml. of 96 per cent alcohol, the mixture was heated for a short time and then placed in the ice-chest. A 25 to 30 per cent yield of the aldehyde was obtained; after recrystallisation from benzene-absolute alcohol mixture, it melted at 147° C. (corr. sealed tube).

Methyl heptenone, $(CH_3)_2C{=}CH(CH_3)_2.CO.CH_3$ (as the p-nitrophenylhydrazone), and hentriacontane, $C_{31}H_{64}$, were isolated from the filtrate. The hydrocarbon is a normal constituent of crude lycopene.

Lycopenal oxime, $C_{32}H_{43}ON$. A solution of 30 mg. of lycopenal in 10 ml. of benzene was mixed with 2 ml. of an alcoholic solution of free hydroxylamine (60 mg.) and the mixture was warmed for 4 hours on a water-bath. It was then diluted with 1 litre of petroleum (b.p. 70° to 80° C.) and the excess of hydroxylamine was removed



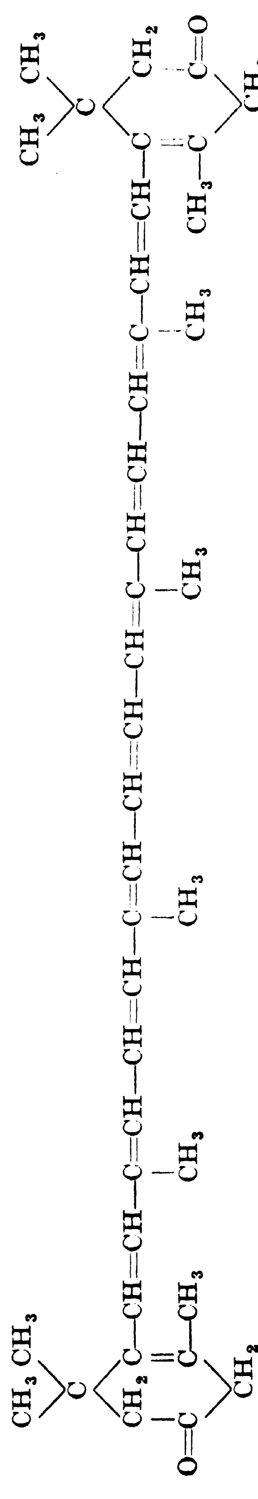
Lycopenal

by washing with water. The solution was poured on to a column of calcium carbonate (Merck's pure precipitated) and the chromatogram was developed with petroleum. The oxime was eluted with chloroform containing 10 per cent of alcohol and crystallised from 96 per cent alcohol. The product weighed 10 mg. and had a melting-point of 198° C. (corr. sealed tube).

Conversion of Rhodoxanthin, $C_{40}H_{50}O_2$, into Zeaxanthin, $C_{40}H_{56}O_2$ (Karrer and Solmssen 4). (See formulæ, Table 6, pp. 30, 31.)

Dihydro-rhodoxanthin, $C_{40}H_{52}O_2$, was first prepared according to the method of Kuhn and Brockmann (9): 46 mg. of rhodoxanthin were dissolved in 10 ml. of pure pyridine and 3 ml. of glacial acetic acid were added. The solution, warmed to 50° C., was treated with 0·5 g. of zinc dust and then filtered. The filtrate was extracted with 15 ml. of petroleum, and the extract when evaporated gave glistening golden-yellow leaflets in a yield of 65 per cent. On recrystallisation from a mixture of benzene and methyl alcohol these had melting-point 219° C.

Sixty-five milligrams of dihydro-rhodoxanthin were dissolved in 15 ml. of benzene, and 20 ml. of absolute isopropyl alcohol were added, followed by 2 g. of aluminium isopropylate. The mixture was heated for 24 hours in a 100-ml. flask fitted with an efficient fractionating column. The temperature at the top of the column was not allowed



Dihydro-rhodoxanthin

to exceed 40° C., and a continuous stream of nitrogen was passed into the flask. The excess of aluminium isopropylate was decomposed with 20 ml. of 10 per cent potassium hydroxide solution and the pigment was extracted with ether. The extract was washed, dried and evaporated. The residue, dissolved in benzene, gave the following chromatogram on a column of calcium hydroxide:

Top:	orange-yellow (510, 480 mμ in carbon disulphide)
	yellow
	yellow (non-uniform)
Bottom:	orange-yellow (515, 485 mμ)

The bottom zone, containing most of the pigment, was eluted with a mixture of benzene and methyl alcohol. The eluate was washed with water and evaporated under reduced pressure, and the dry residue was crystallised from methyl alcohol. A yield of 8 mg. of zeaxanthin of melting-point 204° to 205° C. (uncorr.) was obtained. It had absorption maxima in carbon disulphide solution at 518 and 483 mμ.

D. Examples of the Separation of Carotenoids by Micro-Chromatography

By means of the apparatus shown in Fig. 28, page 66, Willstaedt and With (1) were able to separate several of the polyenes from one another, and occasionally to obtain an estimate of the amount of particular constituents present. It was found that with quantities of less than 30 μg, only a crude estimate of the amount present could be made, whilst amounts of from 30 to 150 μg could be estimated with an error of 20 to 50 per cent. The biggest losses occurred with the polyenes that were most strongly adsorbed. The petroleum solution was introduced into the column by means of a small funnel or a Pasteur pipette, and the column was developed with a mixture of benzene and petroleum in the most suitable proportions. Methyl alcohol was used for elution, and the eluate, after being washed with water to remove the alcohol, was filtered through a small sintered-glass crucible containing a layer of anhydrous sodium sulphate. The filtrate was transferred to a graduated flask and the colour was measured in a Zeiss step-photometer (thickness of solution 0·5 cm., Filter S43).

Separation of Xanthophyll and Lycopene

A mixture of xanthophyll (33 μg) and lycopene (36 μg) was chromatographed on a small column (10 × 1 cm.) of "pure anhydrous" alumina. The chromatogram that developed on washing with a mixture (1 : 4) of petroleum and benzene consisted of a band 1 cm. thick, containing the xanthophyll, and another band 5 cm. below it and 0·5 cm. in thickness, containing the lycopene. The eluates from the two bands were examined colorimetrically and found to contain 15·8 μg and 28 μg of pigment respectively. The losses therefore amounted to 53 per cent of xanthophyll and 22 per cent of lycopene.

Separation of Xanthophyll and β-Carotene

A mixture of 24·5 μg of xanthophyll and 20·5 μg of β-carotene was chromatographed on a small column of alumina, and this was developed with a mixture (1 : 1) of petroleum and benzene. Two zones formed, the upper 1 cm. thick containing xanthophyll, the other—5 cm. below it—2 cm. thick. Each eluate was found to contain 13 μg of pigment, indicating a loss of 45 per cent of xanthophyll and 30 per cent of carotene.

Separation of Lycopene and β-Carotene

The separation was carried out in exactly the same way as in the previous example, with the following results. "Pure anhydrous" alumina was used in experiment (*a*) and "standardised" alumina in experiment (*b*).

(*a*)	Lycopene	Amount taken 43·7 μg	Amount found 37·4 μg	Loss 14%
	β-Carotene	,, ,, 38·4 μg	,, ,, 36·2 μg	,, 6%
(*b*)	Lycopene	,, ,, 58·4 μg	,, ,, 49·3 μg	,, 17%
	β-Carotene	,, ,, 38·4 μg	,, ,, 35·6 μg	,, 7%

Micro-chromatography has also been used in the examination of the polyenes of blood-serum by van Veen and Lanzing.

E. Synthetic Polyenes

Kuhn and Grundmann (7–9) employed chromatography to facilitate working up many of their synthetic products; for example:

(*a*) The diethyl ester of oxalo-octatriene-carboxylic acid,

$C_{14}H_{18}O_5$, obtained by the action of diethyl oxalate on the methyl ester of octatrienic acid, was purified by dissolving the red oil in a mixture of ether and benzene and pouring the solution on to a column of calcium carbonate ("puriss. Merck"). After development of the column with a mixture (1 : 1) of ether and benzene, several coloured bands formed; the filtrate on evaporation yielded a semi-crystalline orange-yellow residue that was recrystallised from 80 per cent methyl alcohol.

(*b*) A mixture of 36·6 g. of octatrienal, 30 g. of crotonaldehyde, 1 ml. of piperidine ("puriss. Merck") and 1 ml. of glacial acetic acid was allowed to stand for 18 hours at 15° to 20° C. in an evacuated, sealed tube. The solid mass was then extracted with 1·5 litres of ether and the insoluble residue was filtered off. The bright red solution was washed several times with water, dried over calcium chloride, and poured on to a column (25 × 7 cm.) of alumina, which was then developed with ether. Unchanged octatrienal gradually passed down the column into the filtrate and was followed by the main reaction product, dodecapentaen-(2 : 4 : 6 : 8 : 10)-al-(1), $CH_3(CH : CH)_5.CHO$, which at first had formed an ill-defined orange-yellow zone. Brownish-red condensation-products remained adsorbed on the upper two-thirds of the column. On evaporation, the filtrate yielded crystalline dodecapentaenal of melting-point 155° C.; when pure it melted at 166° C.

(*c*) Ninety milligrams of the diethyl ester of des-dihydrocrocetin were dissolved in 20 ml. of pyridine and 0·5 ml. of 10 per cent sodium ethylate solution was added. This changed the colour to a deep blue, but, on shaking with air for a few minutes, the colour changed back again to red. The solution was then diluted with 200 ml. of chloroform and washed several times with 2*N*-sulphuric acid and water and the dried solution was then poured on to a column (10 × 2·5 cm.) of alumina.

An orange-yellow zone of the diethyl ester of des-crocetin, $C_2H_5OOC.(CH : CH)_7.COOC_2H_5$, was first formed, then wandered slowly down the column as this was developed with chloroform and finally passed into the filtrate. By-products of the reaction remained adsorbed on the column. The filtrate was evaporated under reduced pressure, and the residue was crystallised from 10 ml. of glacial acetic acid, giving leaflets having a metallic lustre, melting-point 217° C.

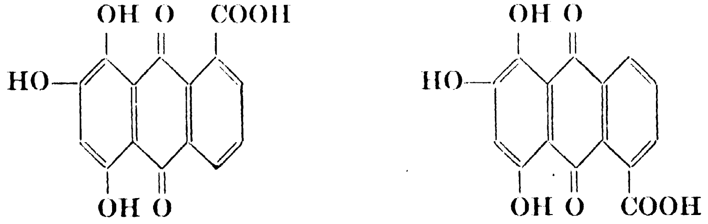
(*d*) The diethyl esters of the oxalo-polyene acids, $HOOC.CO.CH_2.(CH : CH)_n.COOH$, obtained by Grundmann, were purified by chromatographing from ethereal solution on columns of calcium carbonate. The main products were recovered from the filtrates, impurities remaining as narrow dirty-brown bands on the adsorbent.

d-Cumulene is the name given by Kuhn and Wallenfels to a class of synthetic substances, in which a large number of carbon atoms are connected together by an unbroken sequence of double linkages. Thus 1 : 1 : 6 : 6-tetraphenyl-hexapentaene, $(C_6H_5)_2C : C : C : C : C : C(C_6H_5)_2$, was prepared by the action of phosphorus diiodide on tetraphenyl-hexadiin-(2:4)-diol-(1:6). The orange-yellow crystals that were obtained appeared to be homogeneous, but melted over a wide range, viz., from 100° to 125° C. Eight grams of this crude product were therefore dissolved in 500 ml. of benzene and the solution was chromatographed on a column (20 × 5 cm.) of alumina. The cumulene passed into the filtrate, which gave a deep red residue on evaporation; colourless impurities were adsorbed on the column. The residue on recrystallisation from glacial acetic acid-chloroform mixture had a melting-point of 302° C. (after sintering).

6. Naphthaquinone and Anthraquinone Pigments

(*a*) Quinizarin Series

The fungus pigment boletol (from *Boletus satanas* or *B. badius*), belonging to the quinizarin series, was synthesised by Kögl and Deijs and separated from accompanying isoboletol by chromatography. The formulæ for boletol and isoboletol are given below, but it is not yet known which one of these represents boletol and which isoboletol. In any event, the small difference in the two structures is sufficient to produce a considerable difference in adsorption-affinity.



Boletol and isoboletol

Quinizarin-α-carboxylic acid was dehydrogenated to the corresponding quinone with lead tetra-acetate. The product (100 mg.) was heated for 10 minutes with 8 ml. of acetic anhydride and a few drops of conc. sulphuric acid, which resulted in the introduction of two acetyl-groups and one acetoxy-group into the molecule. The solution was filtered from precipitated lead sulphate and the filtrate was cautiously treated with water. The yellowish-brown solid that separated was warmed on the steam-bath with 5 per cent alcoholic potassium hydroxide solution for half an hour; after being acidified, the solution was extracted with ether. The residue remaining after evaporation of the ether was dissolved in alcohol and the solution was chromatographed on a column (35 × 2 cm.) of commercial alumina. The chromatogram that formed on development with a mixture of alcohol, benzene and xylene was composed of the following zones:

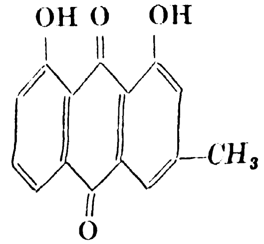
Top:	broad, reddish-brown; containing most of the boletol	
	yellowish-brown; containing a little boletol	
	yellowish-green	containing impurities
	dark green	containing impurities
Bottom:	grey	containing impurities

Elution was accomplished by means of 1 per cent potassium hydroxide solution and the eluate was acidified and extracted with ether. The combined extracts from the top two zones were re-chromatographed, the column being washed successively with alcohol, acetone, and 50 per cent acetone. There formed two easily separated rings, from which the pigments were isolated as before.

For the purification of dichloro-quinizarin quinone, see Criegee.

(b) Chrysarobin Series

Chromatography has also been used for the separation of members of the chrysarobin series. Lederer (private communication) obtained pure pigments by chromatographing the benzene solutions on columns of calcium carbonate or magnesium oxide. In this way chrysophanol (chrysophanic acid), $C_{15}H_{10}O_4$, and one of its monomethyl ethers were separated from one another, though no separation had resulted when other adsorbents were used.

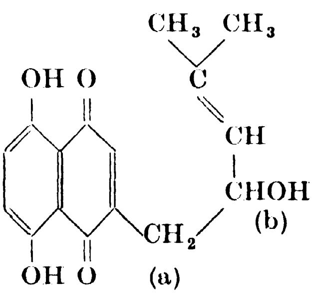


Chrysophanol
(1 : 8-Dihydroxy-3-methyl-anthraquinone)

The pigments of the lichen *Xanthoria parietina*, of which the chief is parietin, can also be separated from one another by chromatography.

(*c*) Alkannin

The naphthaquinone derivative alkannin, $C_{16}H_{16}O_5$, obtained from *Alkanna tinctoria*, is accompanied by another pigment, alkannan, $C_{16}H_{18}O_4$ (3-isohexyl-naphthazarin), which Brockmann (2) first isolated by adsorption on kieselguhr, from its petroleum solution. On developing the column with petroleum, a bright red zone formed underneath the main



Alkannin
(possibly the hydroxyl group is at a, and not at b)

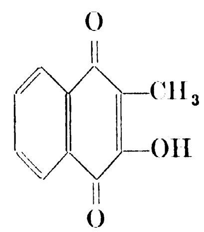
red zone. This was eluted with petroleum containing methyl alcohol and the extract was re-chromatographed. The eluate from the second chromatogram was evaporated and the residue was crystallised from 80 per cent methyl alcohol. The proportion of alkannan to alkannin is 1 : 1,900.

The product obtained by catalytic hydrogenation of alkannin methyl ether (addition of $2H_2$) was purified by filtration of the petroleum solution through a column of calcium carbonate, on which impurities were retained in the

form of reddish-violet zones. The pigment was extracted from the filtrate by means of *N*-sodium hydroxide solution, and the extract was shaken several times with benzene. The extract was washed and evaporated to dryness under reduced pressure, and the petroleum solution of the residue was poured on to a column of calcium carbonate. The residue remaining after evaporation of the filtrate was crystallised from aqueous methyl alcohol at 0° C., giving needles of melting-point 50° to 51° C.

(*d*) Phthiocol

The acetone-soluble fat obtained from heat-killed *Mycobacterium tuberculosis* is of a dark-brown colour, which is partly

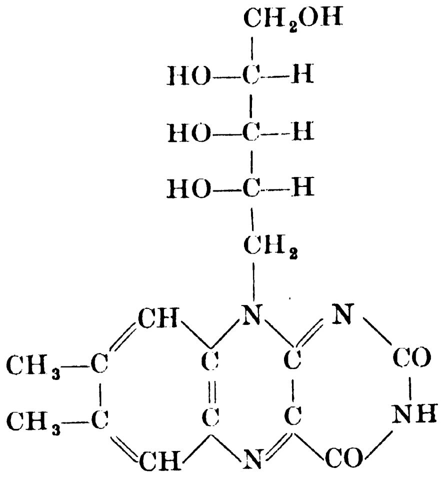


Phthiocol

due to phthiocol. This is adsorbed from benzene solution on Brockmann alumina from which it is eluted by water, alcohol or dilute alkali solution (Wagner-Jauregg).

7. Flavins (Lyochromes)

The flavins that occur in plant and animal tissues have frequently been concentrated by adsorption, but not in the way proposed by Tswett. For examples of the procedure, reference should be made to the work of Ellinger and Koschara, of Kuhn, György and Wagner-Jauregg (1–3), of Kuhn, Wagner-Jauregg and Kaltschmitt (adsorbent: fuller's earth), of Karrer, Salomon and Schöpp (adsorbent: lead sulphide and frankonite), of Karrer and Schöpp (3), and of Greene and Black. Karrer and Schöpp (3) and Euler, Karrer, Adler and Malmberg purified the products they obtained in the above manner, by chromatography after conversion into the acetate. This was dissolved in benzene and poured on to a column of alumina; the chromatogram was eluted with a mixture of methyl alcohol and benzene.



Lactoflavin

In general, flavins can be chromatographed either in aqueous solution or in a non-aqueous medium after acetylation. Both types of procedure are illustrated by the following examples.

Isolation of Flavins, Similar Pigments, and Derivatives

Kuhn and Kaltschmitt employed chromatographic analysis in order to purify the crude flavin obtained from 103 kg. of chopped California lucerne.

The crude product was subjected to a preliminary purification by appropriate methods, and was then acetylated and extracted with dry chloroform, giving a solution that contained 100 mg. of pigment. The chloroform was evaporated off and the brown residue was dissolved in alcohol-free ethyl acetate ; the solution was poured on to a column of Brockmann alumina and the chromatogram was developed with the same solvent. The bright yellow main zone was eluted with a mixture (4 : 1) of ethyl acetate and methyl alcohol. The residue was re-chromatographed, when the pigment was observed to separate into two components.

The upper zone was eluted with water and the eluate was shaken several times with ethyl acetate to remove flavin acetate, leaving in the aqueous phase free flavin formed by hydrolysis. The extract was combined with the material

eluted from the lower zone by means of ethyl acetate-methanol mixture (4 : 1). The solution was evaporated under reduced pressure and the bright yellow residue (30 mg.) was recrystallised twice from absolute alcohol and then twice from boiling water. The acetyl flavin so obtained formed yellow prisms of melting-point 235° C. (decomp.). The lower zone, after elution with ethyl acetate-methanol mixture, still contained pigment, which was eluted by treatment with water. The eluate was shaken with ethyl acetate to separate the flavin acetate from free flavin. The extract yielded a further 17 mg. of the acetate.

Heilbron, Parry and Phipers isolated lactoflavin in the form of its acetyl derivative from the fresh-water alga *Cladophora sauteri*.

By means of chromatography, Strain (2) established the presence in leaves of substances that are strongly fluorescent in ultra-violet light. The adsorbent used was a mixture of magnesia and siliceous earth and the solvent was light petroleum. The nature of the fluorescent substances is not yet known.

Giral reported the presence in certain fluorescent microorganisms (*Bacillus pyocyaneus* Gess., *B. fluorescens liquefaciens* Piork., and *B. putidus*), of an unknown pigment, having properties in some respects resembling the flavins and in others the pterins. It was strongly adsorbed on active charcoal, less strongly on frankonite KL and irregularly on alumina. The charcoal was eluted with 30 per cent acetone, the frankonite with 80 to 90 per cent pyridine, and the alumina with carbonic acid. The fluorescence was bluish-green in ammoniacal solution and an intense violet in mineral acid solution. A red pigment with a blue fluorescence was obtained, possibly identical with pyorubin.

A substance with a blue fluorescence was isolated by Euler and Brandt (1, 2) from corpus luteum; its relationship to lactoflavin is not yet clear. The material was extracted with acetone and then with 70 per cent acetone, and the combined extracts were concentrated to one-third their volume and shaken, first with ether and then with chloroform, to remove impurities. The aqueous solution that remained was chromatographed on frankonite, giving a chromatogram composed

of four zones. On development with a weakly alkaline (*p*H 9) solution, the fluorescent substance washed through into the filtrate.

Synthetic Flavins

Kuhn and Weygand (1) separated chromatographically the by-products formed during the synthesis of 6 : 7-dimethyl-9-*n*-amyl-flavin. A solution of dry 1 : 2-dimethyl-4-amino-9-(*n*-amyl-amino)-benzene in glacial acetic acid was heated for a few minutes with excess of alloxan. After dilution with water, the flavin was quantitatively extracted with chloroform, and the extract was dried and distilled. The residue was triturated with alumina and the mixture was introduced on to a column of alumina. On washing with a mixture of xylene and methyl alcohol, the impurities were quickly carried through, but the flavin passed only slowly down the column.

Kuhn and Rudy purified the synthetic 6 : 7-dimethyl-flavin-9-acetic acid methyl ester by adsorption from chloroform solution on a column of alumina. Elution was effected by means of a mixture (10 : 1) of chloroform and methyl alcohol. 6 : 7-Tetramethylene-9-*l*-araboflavin-tetraacetate was purified in a similar manner. The flavin was in this instance washed out of the column on developing with chloroform, whilst the dark-coloured by-products remained adsorbed. The filtrate was evaporated and the residue was crystallised twice from 15 per cent alcohol, giving orange-yellow star-like clusters of melting-point 243° C. The same procedure is applicable also to 6 : 7-trimethylene-9-*l*-araboflavin-tetraacetate, which was simply recrystallised from water (Kuhn, Vetter and Rzeppa).

For intermediates in the flavin-synthesis, see page 205.

The Flavins (Lyochromes) of Urine (Koschara, 1–3)

(*a*) Preparation of Uroflavin from Male Urine

The so-called " uroflavin," which resembles lactoflavin in most of its properties, consists partly of lactoflavin and partly of a lyochrome containing a higher proportion of oxygen. It was isolated from 5,000 litres of urine by the following procedure :

CONCENTRATION: The urine was treated as described in the section on "uropterin" (see p. 175) up to the point at which the so-called purine fraction (1·2 kg.) was filtered off. The filtrate from this was treated with 12 litres of a solution containing 600 g. of lead acetate and 420 g. of lead hydroxide and the precipitate so formed was allowed to settle overnight. The super-natant liquid was decanted and the residue was filtered and sucked as dry as possible. The filtrate was treated with 1 to 2 litres of 10 per cent lead acetate solution and then saturated with hydrogen sulphide. The precipitated lead sulphide was allowed to settle, the super-natant liquid was decanted, and the residue was filtered off and washed with a little water. The lead sulphide precipitate was eluted with 50 litres of a mixture of water, pyridine and glacial acetic acid in the proportion of 9 : 1 : 0·2. The eluate was concentrated under reduced pressure to a volume of 8 litres, and the concentrate was continuously extracted with ether for 24 hours. The aqueous solution was then filtered from a small amount of precipitate and concentrated to 4·2 litres. This solution contained 700 mg. of lyochromes, about 100 mg. having been lost.

FRACTIONATION BY CHROMATOGRAPHY was carried out in two stages, using "floridin-bleach-earth XXF." In the first chromatogram aquoflavin (see p. 168) washed through the column on developing with water, and the adsorbed lyochromes were then eluted by washing with a mixture (3 : 1 : 1) of methyl alcohol, pyridine and water. Strongly coloured impurities remained adsorbed on the column. The residue from the eluate was chromatographed on a second column and the chromatogram was well washed with water. The lyochromes were again eluted by washing the column with a mixture of methyl alcohol, pyridine and water; the eluate, on being concentrated, yielded crude uroflavin, which was recrystallised twice from *N* acetic acid, giving 220 mg. of pure material in the form of small needles.

Two coloured zones ("third" and "fourth" urine-lyochromes) remained on the second column, and these were eluted by 5 per cent pyridine; purification was effected by further chromatographic treatment, but the investigation of the pigments has not yet been completed.

A blue-fluorescing pigment was isolated by chromato-

graphing the aqueous solution of the lyochromes on coarsely powdered " bleach-earth XS " instead of on the "bleach-earth XXF " used in the initial treatment. The new pigment is so strongly held on the latter adsorbent as to render fractionation impossible.

Chromatography has thus established the presence of at least four lyochrome pigments in urine. These are adsorbed on the column in the following order :

Top :	" third " urine-lyochrome
	" fourth " urine-lyochrome
	Uroflavin (mixture : 95 per cent of the total lyochromes)
Bottom :	Aquoflavin (readily washed through with water)

(*b*) Aquoflavin and Lumi-aquoflavin (Koschara, 2)

Aquoflavin, as indicated above, is washed off the column with water. The filtrate was concentrated and treated with one-fifth of its volume of 2 *N* sulphuric acid and adsorbed once again. On developing the column with water, some impurities were first removed and a yellowish-red fluorescent zone of aquoflavin was gradually formed. On further washing, this passed through into the filtrate, leaving a brownish-coloured pigment on the column. Ten millilitres of solution containing 1·3 mg. of aquoflavin were transferred to a Petri dish resting on a white background and treated with 2 ml. of 2 *N* sodium hydroxide solution. The solution was irradiated for 55 minutes with a 75-watt metal filament lamp placed 10 cm. from the dish. Four millilitres of sulphuric acid were then added and the solution was shaken with chloroform. The aqueous layer was separated, freed from traces of chloroform by warming under reduced pressure and then chromatographed on a column (2·8 × 2 cm.) of " bleach-earth XXF." Lumi-aquoflavin was adsorbed at the top of the column ; unlike aquoflavin, it was not eluted on washing with water, but was removed from the column by means of a 5 per cent solution of pyridine.

(*c*) Quantitative Estimation of Flavins (Lyochromes) in Urine (Koschara, 3)

Two hundred and fifty millilitres of urine were concentrated to 50 ml. under reduced pressure after the addition of a little octyl alcohol. The concentrate was treated with 0·5 ml. of

conc. hydrochloric acid (the slight precipitate that formed being ignored) and chromatographed on a column (4 × 2 cm.) of "bleach-earth XS" that had first been treated with acid. The column was washed with 20 ml. of 0·1 N hydrochloric acid and then eluted by washing with a mixture (9 : 1 : 0·2) of water, pyridine and acetic acid, until the filtrate came through colourless, whereupon it was concentrated under reduced pressure with the addition of a little barium carbonate towards the end. The solution was filtered and the filtrate (20 ml.) was acidified with five drops of glacial acetic acid and then cooled in ice. Six drops of cold, saturated potassium permanganate solution were added and, after 1 minute, the excess was destroyed by the addition of one drop of 3 per cent hydrogen peroxide solution.

After the oxidation, the presence of lyochrome was indicated by a green fluorescence in ultra-violet light. (If the fluorescence is blue, further purification is useless, as the flavin content is less than 10 μg per cent.) The material was given a further adsorption on a 2 cm. column of "bleach-earth XS." This was washed first with water and then with 2 per cent pyridine solution, which removed a blue-fluorescing substance; the flavin was eluted by washing with a mixture (3 : 1 : 1) of methyl alcohol, pyridine and water. The column was finally washed with a mixture of pyridine and acetic acid in order to check the completeness of the elution. The eluate was concentrated to 5 ml. and transferred to a step-photometer.

This procedure is also applicable to other liquids besides urine.

For the estimation of lyochromes in concentrates of urine (4·2 litres from 5,000 litres), see Koschara (3).

8. Pterins

The pterins form a group of widely distributed pigments, occurring especially in insects; because of their high nitrogen contents, they are believed to have a pyrimidine- or purine-type of structure. (For definition, see Wieland and Schöpf; Schöpf and Becker.) The following data on the chromatography of insect-pterins, and in particular of the butterfly pterins, are taken from the comprehensive researches of Schöpf and Becker, and Becker and Schöpf. Koschara's isolation of

uropterin from human urine by chromatography is described on page 175.

The adsorption behaviour of the most important pterins, which are probably mixtures, can be summarised as follows :

Erythropterin is adsorbed more strongly from very dilute solutions in aqueous or methyl alcoholic 0·004 *N* hydrochloric acid than xanthopterin and remains on the column as a narrow band. Xanthopterin on the other hand is retained very feebly from aqueous acid solution, and only slightly less feebly from methyl alcoholic acid solution. The presence of erythropterin in a preparation of xanthopterin can be detected by pouring the aqueous hydrochloric acid solution on to a column of alumina (a micro-chromatogram can be employed with advantage ; see p. 66), when a red erythropterin band, possessing a rich brown fluorescence in ultra-violet light, is formed. In the same way, xanthopterin can be detected in erythropterin by chromatographing from methyl alcoholic hydrochloric acid solution ; in this instance the xanthopterin forms a narrow yellow band with a yellowish fluorescence below the red erythropterin zone. The pigments are gradually destroyed by adsorption on alumina from acid solution ; thus on developing the chromatogram with aqueous or methyl alcoholic hydrochloric acid, the xanthopterin band grows fainter and fainter, and ultimately disappears altogether. The filtrate has a blue fluorescence.

Erythropterin is adsorbed on " floridin XXF " as well as on alumina from very dilute solution in 0·004 to 0·002 *N* hydrochloric acid, and on frankonite from a mixture of acetic acid and sodium acetate solution. The pigment is eluted from alumina with 0·5 *N* ammonia solution or with 4 per cent aqueous pyridine. Erythropterin apparently shows evidence of heterogeneity when adsorbed on frankonite KL from solution in acetic acid + sodium acetate solution, but lack of uniformity in the adsorbent itself may be the true explanation of the phenomenon.

Xanthopterin is adsorbed on alumina, frankonite, or floridin XXF from neutral or slightly acid aqueous solution, but it is washed quickly through a column of alumina with slightly stronger acid. Like erythropterin, it is adsorbed on purified floridin from 0·004 to 0·002 *N* acid solution. It is

firmly held on a column of frankonite KL, when a solution in acetic acid or a neutral solution is used, and a very concentrated adsorbate is then formed at the top of the column. After being washed with water, the chromatogram is eluted with 0·5 N ammonia solution. Xanthopterin is also well adsorbed on alumina from its solution in anhydrous methyl alcohol containing 0·01 per cent of hydrogen chloride ; the colour of the pigment zone, however, gradually disappears on prolonged development. Xanthopterin, unlike erythropterin, is not adsorbed on barium sulphate from methyl alcoholic pyridine solution.

Chrysopterin is adsorbed more strongly than xanthopterin on alumina from 0·004 N hydrochloric acid, giving a band with a yellowish-green fluorescence. Both the hydrochloric and the acetic acid solutions show a violet-blue fluorescence. Adsorption from methyl alcoholic hydrochloric acid solution is not so characteristic. The colourless, non-fluorescent guanopterin is, like xanthopterin, adsorbed from dilute solution in very weak acid on frankonite KL, from which it is eluted by 0·5 N ammonia solution.

For further details concerning the adsorption behaviour of the pterins, reference should be made to the original papers.

Preparation of Crude Xanthopterin

The wings (32·2 g.) of 2,000 butterflies of the species *Appias nero*, a pierid from Java, were extracted with ether to remove the fat, after a preliminary treatment with alcohol. The material was then extracted with ammonia solution and the solution was fractionally precipitated by the addition of hydrochloric acid. Some of the pigments remained in the acid mother-liquors (5 litres) and were concentrated by chromatographic adsorption. The solution was treated cautiously with saturated sodium acetate solution until the reaction to congo red indicated an acidity corresponding with that of 0·003 N acid, and the solution, without being filtered, was drawn through a 20-mm. layer of alumina (" puriss. Merck "), which was then washed with a little water. The resulting chromatogram was composed of a deep reddish-violet zone 1 mm. thick at the top and a deep reddish-brown zone of erythropterin

underneath this, whilst the rest of the adsorbent was coloured pale pink.

The pale yellow filtrate, containing the xanthopterin, became turbid on standing for 24 hours and was run through a 10-mm. layer of alumina, the brown adsorbate being rejected. The filtrate was completely neutralised by the addition of 25 ml. of saturated sodium acetate solution and drawn through yet a third layer (10 mm.) of alumina. The bright yellow filtrate had a yellowish-green fluorescence in ultra-violet light and soon developed a lemon-yellow turbidity. It was now poured on to a 10-mm. column of frankonite KL on which the pigment was adsorbed as a band 5 mm. thick. The filtrate was rejected.

The deep yellow zone was separated from the rest of the column, washed once with water, and centrifuged, and then eluted with three 100-ml. portions, followed by five 50-ml. portions, of 0·5 *N* ammonia solution. The turbid alkaline solution was filtered through asbestos wool, which retained a greyish-violet substance, and the clear, golden-yellow solution was precipitated with acetic acid. The precipitate was centrifuged off, washed twice with water, and then extracted six times with 100-ml. or 50-ml. portions of 0·25 *N* hydrochloric acid. The combined extracts, on being neutralised with saturated sodium acetate solution, deposited a bright golden-yellow precipitate of crude xanthopterin. This was centrifuged off and washed with water until a colloidal solution seemed about to form. The yield was 1·3 g.

The wing-pigments of the cabbage-white butterflies (*Pieris brassicæ* and *P. napi*) consist of the white leucopterin, a water-soluble pigment, a yellow pigment, and, according to Wieland and Kotzschmar, a green chromoprotein. The chromatogram formed on alumina from aqueous solution consisted of an upper yellow and a lower green zone.

Micro-Chromatography of Pterins

Becker and Schöpf used the micro-adsorption apparatus illustrated in Fig. 67, page 322, for their investigations on the pterins. The amount of pigment with which they worked was very small indeed, being of the order of a few μg only. The

following examples illustrate the application of the method to pure solutions of pterins and also to crude insect extracts.

Xanthopterin is retained on a column of alumina as a sharply defined yellow zone, with a bright yellowish-green fluorescence, when a solution in methyl alcoholic hydrochloric acid is used. The test can be successfully applied in those instances where xanthopterin occurs as a minor constituent of a mixture of several pterins. It is important that the column should not be developed more than necessary, and washing should be discontinued when, for example, the band has moved a distance down the column equal to three or four times its thickness, as otherwise there may be serious loss by destruction. If the amount of solvent used is thus cut down to a minimum, it is possible to estimate the amount of pigment present from the thickness of the band, the limit being 1 μg. From columns 1 and 3 of Table 16 it will be seen that the thickness of the xanthopterin band is approximately proportional to the amount of pigment present. In the experiments from which these data were obtained, the columns were not developed at all, and the total volume of solution used was only 5 ml.

TABLE 16

ESTIMATION OF XANTHOPTERIN IN ADMIXTURE WITH ERYTHROPTERIN, USING A MICRO-COLUMN OF ALUMINA

5 ml. of 0·01 per cent Methyl Alcoholic Hydrochloric Acid contain		Thickness of Xanthopterin Zone (mm.)	Appearance of Chromatogram
Xanthopterin (μg)	Erythropterin (μg)		
0	40	—	Broad orange-red zone
5	0	2	Xanthopterin band (yellow),
			yellowish-green in ultra-
10	0	4	violet light
0·4	40	0·2	Top red, yellow below (only
			detectable in ultra-violet
2	40	nearly 1	light)
40	2	15	Orange-red erythropterin
			band above the xantho-
40	4	15	pterin zone

Xanthopterin is not so well adsorbed on alumina from aqueous 0·004 *N* hydrochloric acid as it is from methyl alcoholic hydrochloric acid. The presence of 0·5 to 1 μg of xanthopterin on a layer of adsorbate 0·5 to 1 cm. thick cannot be detected in ordinary daylight, but its presence is indicated by a bright yellowish-green fluorescence in ultra-violet light; when a larger quantity than this is present, almost all the column fluoresces. If appreciable amounts of pigment are available, the procedure used by Koschara (4, 5) in the isolation of uropterin can be adopted, viz., adsorption from a buffer solution on frankonite and, after washing, elution with another buffer solution (see p. 175).

There exists the possibility of confusing xanthopterin with lactoflavin when the pigment is adsorbed on alumina, though the fluorescence of the adsorbate is slightly different in the two instances. Lactoflavin has a warmer, almost orange-yellow, shade, whilst xanthopterin has a yellowish-green tinge.

Erythropterin can best be detected by adsorption on alumina from 0·004 *N* hydrochloric acid, as a sharp zone is only formed from aqueous solutions. The colour of the adsorbate is orange-red, but it appears velvety-brown in ultra-violet light. The limit of the test is 5 μg of pigment in 5 ml. of solution, this forming a band 1 mm. thick in the micro-adsorption tube.

The Detection of Pterins in Insects by Micro-Chromatography

The method used by Becker and Schöpf was to extract the pigments twice with 0·5 *N* and then once with 2 *N* cold ammonia solution and, after centrifuging the combined extracts, to dry the solutions in a desiccator containing potash and sulphuric acid.

Twenty disc-shaped pieces, cut by means of a cork-borer from the wings of 5 male brimstone butterflies (*Gonepteryx rhamni*), were extracted as described above and the dry residue was dissolved in 1 ml. of 0·004 *N* aqueous hydrochloric acid. The solution was poured on to a micro-column of alumina, which was washed with 0·75 ml. of acid. The chromatogram so formed had the following appearance:

Top : 3 grey : impurities
1·5 orange-red : erythropterin (brown fluorescence in ultra-violet light)
4 almost colourless : decomposition product (blue fluorescence)
Bottom : yellowish-green : xanthopterin

The extract from the wings of female butterflies gave a somewhat different chromatogram.

The abdominal integuments (25 mg.) of hornets (*Vespa crabro*) were extracted with two 1-ml. portions of 0·5 *N* ammonia solution and the residue left on evaporation of the extract was triturated with 1 ml. of 0·01 per cent methyl alcoholic hydrochloric acid. The extract was cautiously decanted through a filter and the filtrate was chromatographed on alumina. A zone of xanthopterin 7 mm. thick formed 5 mm. below the top of the column ; it had a bright yellowish-green fluorescence. The insoluble residue was then treated with 0·004 *N* aqueous hydrochloric acid and this solution gave the following chromatogram :

Top : grey : impurities
2·5 blue fluorescence : decomposition product of the adsorbed xanthopterin
Bottom : occupying the rest of the column, yellowish-green fluorescence : xanthopterin

The excreta of *Gonepteryx rhamni* and of *Vespa crabro* contained no pterins (Becker).

Isolation of Uropterin from Human Urine

Uropterin is a yellow pigment, closely related to the xanthopterin of *Gonepteryx rhamni* ; it gives a positive murexide test and occurs in urine at a dilution of 1 : 1,000,000. The pigment was isolated by Koschara (5) and the isolation affords a striking example of the value of using fluorescence analysis in conjunction with adsorption analysis.

CONCENTRATION OF THE PIGMENT BY CHROMATOGRAPHY : Two hundred litres of urine were collected daily in 3 litres of 25 per cent hydrochloric acid. The solution was stirred for 10 minutes with 4 kg. of " bleach-earth XXF," and the adsorbate was allowed to settle and transferred to a filter and sucked as dry as possible. It was eluted, without being washed,

with 10 to 15 litres of 20 per cent aqueous pyridine solution. The eluates from 5,000 litres of urine were concentrated under reduced pressure to about 60 litres and the precipitate that formed (purine fraction = 1·2 kg.) was filtered off. Each 50-g. portion of this residue was shaken with 500 ml. of *N* sodium hydroxide solution for 1 hour and the insoluble material was centrifuged off and re-treated. The alkaline solution was added to 1 litre of boiling 2 *N* hydrochloric acid and the mixture was kept at 60° to 70° C. until the coloured, amorphous precipitate became crystalline (2 to 3 minutes). After cooling, the crystals (uric acid 30 g.) were filtered off and the filtrate (xanthine bases) was stirred with 170 g. of " bleach-earth." The suspension was filtered and the adsorbate was washed until free from acid and then eluted with 1·2 litres of 5 per cent pyridine solution. The eluate was concentrated to a volume of 400 ml. and 60 ml. of conc. ammonia and 20 g. of ammonium chloride were added. The solution was stirred with 30 ml. of 5 per cent silver nitrate solution and the xanthine bases were centrifuged off. The mother-liquors were treated with 240 ml. of silver nitrate solution and, without being washed, the resulting precipitate, containing the pigment, was stirred on the centrifuge with *N* hydrochloric acid, in order to dissolve the pigment. The acid solution was stirred with " bleach-earth " and the adsorbate was washed free from acid and eluted with pyridine. The pyridine was distilled off and the pyridine-free pigment solution was dissolved in 500 ml. of phosphate buffer solution of *p*H 7·6.

ADSORPTION ANALYSIS. Ninety grams of frankonite KL were stirred with 250 ml. of phosphate buffer solution of *p*H 7·6, and a column of adsorbent was built up on a buchner funnel (diameter 12·3 cm.) with the aid of gentle suction. At least 1 hour must be allowed to elapse between the preparation of the suspension and its transfer to the funnel. The solution of the pigment in the phosphate buffer solution (500 ml.) was then poured on to the column. At the beginning of the adsorption a vacuum equivalent to 5 cm. of water was applied, and was slowly increased to 30 cm. during the filtration. Full vacuum was applied towards the end of the process. After all the pigment solution had been added, the adsorbate was washed with 250 ml. of phosphate buffer solution (*p*H 7·6) and then

with 800 ml. of 1/15 *M* disodium phosphate solution (*p*H 8·3). Although only a small amount of impurity was thereby removed, this intensive washing was necessary in order to prepare the column for development.

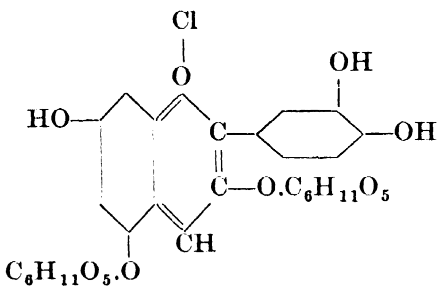
The column was then washed with borate buffer solution of *p*H 9·2 ; after the addition of about 300 ml. of this solution, the pigment began to make its appearance in the filtrate. It was rather difficult to determine the point at which the elution of the uropterin was complete, but the procedure was facilitated by observing the fluorescence of the filtrate. The uropterin usually comes through in the first 200 ml. of solution. The pigments that subsequently ran through the column were characterised by a strong reddish fluorescence, especially when viewed in ultra-violet light. On standing overnight in the ice-chest, the uropterin solution deposited yellow crystals of uropterin salt containing a small amount of " bleach earth " (30 mg.).

The crystalline material was extracted with *N* ammonia solution and silver nitrate solution was added. The precipitated silver salt was washed once with *N* ammonia solution and once with water, then decomposed with 10 ml. of *N* hydrochloric acid and centrifuged, the silver chloride being washed with 5 ml. of hydrochloric acid. The combined acid solutions were cooled in ice and neutralised by adding a saturated solution of sodium acetate. The amorphous, reddish-yellow precipitate, after being allowed to stand for 1 hour, was centrifuged off and washed three times with 5 ml. of ice-cold water, giving a yield of 40 mg. of crude pigment. Of this 7·5 mg. were dissolved in 10 ml. of *N* ammonia solution, 10 ml. of *N* acetic acid were added and the solution was heated and filtered. On standing, the filtrate deposited reddish-yellow spherical crystals, and a greenish crystalline crust formed on the surface. The yield of crystalline pterin was 3·8 mg.

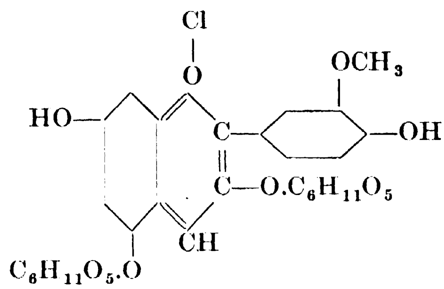
9. Anthocyanins

The chromatography of anthocyanins was attempted by Tswett himself (1, 9), though not with much success, because these pigments are sparingly soluble in petroleum, carbon disulphide and similar solvents. The recent work of Karrer

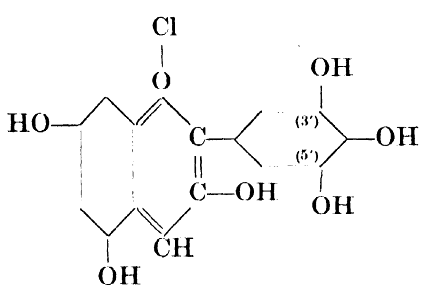
and Strong (1) and of Karrer and Weber has shown how this difficulty can be overcome. Karrer and Strong (1) ran an aqueous solution of the pigment through a column of alumina that had been weakly activated with tap water according to the method of Ruggli and Jensen (1).



Cyanin chloride



Pæonin chloride



Delphinidin chloride

It should be noted that a homogeneous anthocyanin can give rise to red, blue and bluish-green zones, as the basic alumina converts a portion of the oxonium salt into the carbinol base and phenol betaine, so that the same type of pigment is obtained from the different parts of the column. On the other hand, a mixture of pigments may give rise to only one zone in a chromatogram. Thus Price and Robinson attempted in vain to purify the crude preparation obtained from *Bougainvillæa glabra* by adsorption on a column of alumina. Although two well-defined bands were formed, the upper one purple and the lower yellow, the latter was shown to contain a mixture of a glycoside with its aglycone.

The experiments of Karrer and Weber showed that the pigment of the black mallow, the so-called althæin, was actually a mixture of the monoglycoside of delphinidin-3′ : 5′-dimethyl

ether (syringidin), delphinidin-3′-monomethyl ether and delphinidin. The first-named glycoside is apparently identical with œnin, the pigment of wine. The fractions obtained from the chromatogram were further purified *via* the picrates.

(*a*) **Separation of Cyanin- and Pæonin-Chloride** (Karrer and Strong)

Pæonin is a methyl ether of cyanin and is much less strongly adsorbed than the pigment containing three free hydroxyl groups. Crude pæonin chloride, obtained from the red flowers of the peony, was freed in the following manner from the cyanin chloride that accompanied it and was thus obtained in a high degree of purity. The colour of a dilute alcoholic solution of the pigment thus purified was not changed to violet on the addition of ferric chloride solution ; this reaction, hitherto given by specimens of pæonin prepared without chromatography, depends therefore on the presence of cyanin.

The solution of 1·5 g. of crude pæonin in 200 ml. of warm water was adsorbed on alumina and the chromatogram was developed with water. A purple band formed at the top and a bright blue band at the bottom, whilst a considerable amount of pigment (pæonin chloride) was washed through into the filtrate. The washing was continued until the filtrate was colourless. The upper anthocyanin band was eluted ten to twelve times, and even then incompletely, with warm water containing a little hydrochloric acid, and the eluate was concentrated under reduced pressure to about 20 ml. The pigment that precipitated was filtered, washed with a little water, acetone and ether and redissolved in water, and the solution was re-chromatographed. The second chromatogram had a similar appearance to the first, the upper band was eluted in like manner and the eluate was concentrated. The concentrate was heated for 10 to 15 seconds with 20 ml. of 0·5 *N* hydrochloric acid, whereupon the precipitate went into solution. After being quickly filtered, the solution was allowed to stand at 0° C., when it yielded crystals, in which methoxyl groups were still present. The substance was therefore subjected to a third chromatographic separation. The crystalline material (110 mg.) obtained from the third eluate was only sparingly soluble in hot 0·5 *N* hydrochloric acid ; the insoluble residue

was accordingly extracted with 10 ml. of boiling water and the filtrate was treated with 0·6 ml. of conc. hydrochloric acid. On standing in the ice-chest 76 mg. of pure methoxyl-free cyanin chloride crystallised out.

Pæonin chloride was obtained from the combined filtrates from the first two chromatograms. These were concentrated to 20 ml. under reduced pressure at 30° C. and the resulting precipitate was filtered off and heated for a short time with 50 ml. of 0·5 *N* hydrochloric acid. The crystalline product was washed with water, acetone and ether and was then obtained in the form of fine red needles. (Yield 0·55 g.)

(*b*) Separation of "Althæin" into its Components (Karrer and Weber)

An aqueous solution of 2 g. of althæin chloride was drawn through a column (80 cm.) of hydrated gypsum. One pigment fraction was adsorbed at the top, whilst another washed through into the filtrate, which was concentrated under reduced pressure at 30° C. (Fraction I). The adsorbed pigment was eluted with warm, very dilute hydrochloric acid. After neutralisation, the eluate was concentrated and the concentrate was re-chromatographed on gypsum. Two distinct zones, different in colour, were formed in the top part of the column (upper, Fraction II; lower, Fraction III), but no pigment appeared in the filtrate. The two zones were separately eluted as before and the eluates were concentrated under reduced pressure at 30° C.

The three fractions thus obtained were precipitated with picric acid solution and the picrates were crystallised several times from boiling water. The crystalline anthocyanin chlorides were obtained by dissolving the picrates in 2 per cent methyl alcoholic hydrochloric acid and precipitating with ether.

Fraction I was obtained as the analytically pure monoglycoside of a delphinidin-dimethyl ether and was identified as œnin (11·5 per cent of OCH_3). It dissolved in water to give a red solution that did not change colour on the addition of ferric chloride solution. Fractions II and III were not perfectly homogeneous; they appeared to contain a monoglycoside of delphinidin-3′-methyl ether with some delphinidin glycoside present as an impurity, whereby the methoxyl content was

somewhat depressed (found 5 to 6 per cent of OCH_3). The ferric chloride test was positive.

For the separation of derivatives of flavanone, reference should be made to the work of Fujise and Nagasaki.

Pigments of Wine. Detection of Sophistication

The following work of Mohler and Hämmerle (1, 2) illustrates the use of chromatography in the examination of foodstuffs (see also pp. 289 and 302).

The experiments were carried out with the apparatus illustrated in Fig. 15, page 61, using Brockmann alumina as adsorbent. Absolute alcohol was first added to the wine to bring the alcohol concentration up to 50 per cent, 35 ml. of alcohol for example being added to 40 ml. of wine. The column was then saturated with 50 per cent alcohol (20 ml.). A

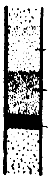


FIG. 38.—Chromatogram of a red wine.

genuine red wine yielded the chromatogram illustrated in Fig. 38, in which Zone 3 was produced by the action of the alkaline adsorbent; the filtrate had a very faint bluish tinge. Zones 1 and 2 were combined and eluted five times with a 2 per cent solution of tartaric acid in 50 per cent alcohol, and the colour of the eluate was measured colorimetrically and spectrophotometrically.

The detection of added coal-tar dye (Bordeaux red) was accomplished in a similar experiment. The added pigment passed through the column and was determined quantitatively in the filtrate. An important consideration that must be borne in mind is the acidity (expressed as tartaric acid per cent) of the wine, which must be adjusted if necessary. If, for instance, the concentration of the added Bordeaux red is 0·025 per cent, then the concentration of tartaric acid must not exceed 5 per cent or the synthetic pigment will also be adsorbed, forming

a zone just below the naturally occurring anthocyanin. The behaviour of the pigment on the chromatogram is also influenced by the amount of tannic acid present (see Table 17).

TABLE 17

CHROMATOGRAPHIC BEHAVIOUR OF BORDEAUX RED IN 50 PER CENT ALCOHOLIC SOLUTION, IN THE PRESENCE OF TARTARIC ACID AND TANNIN

Solution contains			Result
Tartaric Acid (%)	Tannin (%)	Dye (%)	
5	0	0·025	No adsorption
5	1	0·025	Only tannin adsorbed
5	2·5	0·025	Dye and tannin adsorbed almost quantitatively; only in the first experiment was elution obtained with 50 per cent alcohol
5	4	0·025	
10	4	0·025	
10	4	0·050	Dye and tannin adsorbed; dye scarcely eluted with 50 per cent alcohol

In one instance the adulteration of red wine by white wine had been masked by the addition of a coal-tar dye, as was shown by means of chromatography. Zones 1 and 2 (Fig. 38, p. 181) were abnormally feeble, and the synthetic pigment was recovered in the filtrate.

10. OTHER NATURAL PIGMENTS

(For indirubin, see p. 273.)

Pigments of Fungi

Willstaedt (6, 7) isolated two pigments from the fungus *Lactarius deliciosus* by means of chromatography; the main pigment was lactaro-violin, $C_{15}H_{14}O$, and the other pigment was a blue liquid hydrocarbon, azulene, $C_{15}H_{18}$, which can also be obtained from oil of chamomile by adsorption on alumina from light petroleum solution ("chamazulene"). This latter pigment is formed by the action of air during the process of working up the fungus.

Lactaro-violin

Three kilograms of the powdered fungi were allowed to stand for a day with 2 litres of alcohol and separated from the liquid by filtration through cloth ; the extract was expelled by expression. The extraction was repeated with a further 1 litre of alcohol. The extract was run through a folded filter and half a volume of ether and water was added to the filtrate. The red ethereal solution was allowed to stand overnight with one-twentieth of its volume of alcoholic sodium hydroxide solution, which caused the separation of a colourless, crystalline mass. The ethereal solution was decanted off, washed free from alkali, and dried. The residue left on evaporation was dissolved in light petroleum and the filtered solution was poured on to a column of Brockmann alumina ; the chromatogram was developed with a mixture (1 : 5) of benzene and light petroleum. A narrow greenish-grey band formed at the top of the column and the red main zone below it, from which on development a pure blue band separated. The red zone was eluted with a mixture of light petroleum and methyl alcohol and the product obtained from the eluate was re-chromatographed. The reddish-violet eluate obtained from the second chromatogram was washed with water, concentrated, and allowed to stand in the ice-chest. Lactaro-violin crystallised out ; after being recrystallised from light petroleum containing a little benzene, it was obtained in the form of rods of melting-point 53° C.

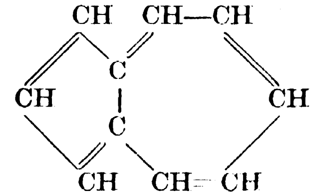
Azulenes

The powdered fungus, after being twice extracted with alcohol (see above), was allowed to stand for one day with acetone, separated from the liquid and again extracted with acetone. Water and petroleum were added to the deep blue solution, and the aqueous layer was separated off and extracted with petroleum. The blue petroleum extracts were combined and washed free from acetone. After being dried, the solution was poured on to a column of alumina, and the following chromatogram resulted :

Top :	a number of narrow bands : violet, brownish-yellow, black, purple (lactaro-violin), olive-green
	a broad, light blue band : " azulene II "
	a broad, intense blue band : lactar-azulene (main fraction)
Bottom :	an orange band that readily passed into the filtrate

The main zone was eluted with a mixture of light petroleum and methyl alcohol, and the latter was removed from the eluate by washing with water. The light petroleum was distilled off in an atmosphere of nitrogen and the residual lactar-azulene, $C_{15}H_{18}$, was distilled in vacuo over a range of 155° to 160° C. at 2·5 to 3 mm. pressure. When the distillate was treated with 1 : 3 : 5-trinitrobenzene in alcoholic solution black needles were formed ; these consisted of the addition compound of trinitrobenzene and lactar-azulene. Thirty kilograms of the fresh fungus yielded 4·5 g.

The crude azulenes obtained by dehydrogenation of sesquiterpenes with sulphur or selenium can similarly be purified by chromatography (Pfau and Plattner). For example, the mixture obtained by reacting cyclopentano-cycloheptanone with magnesium and bromobenzene was dehydrogenated with sulphur at 170° to 240°/100 mm. for a period of three-quarters of an hour, and the distillate was twice chromatographed on alumina from light petroleum solution. Brownish-coloured impurities remained adsorbed on the column, whilst the blue azulene ran through into the filtrate, together with colourless hydrocarbons (Willstaedt 6, 7).



Azulene : Bicyclo-(0 : 3 : 5)-decapentaene-(1 : 3 : 5 : 7 : 9)

The parent substance of the group, azulene itself, $C_{10}H_8$, was obtained by Plattner and Pfau in the following way. Cyclopentano-cycloheptanol was dehydrogenated, as described above, and the pigment was isolated as its addition compound with trinitrobenzene. An attempt to split this by fractional sublimation failed, for it was found impossible to remove the whole of the trinitrobenzene by this method. The separation was, however, accomplished by chromatography. The addition compound (0·4 g.) was put on to a column (18 × 1·7 cm.) of Brockmann alumina, which was then washed with 50 ml. of

a 1 : 1 mixture of cyclohexane and benzene, followed by 30 ml. of cyclohexane. The trinitrobenzene remained on the column as a brown zone, whilst the azulene passed right through it. The blue filtrate was distilled with an efficient fractionating column to recover the cyclohexane, and the remaining solvent was distilled off. A yield of 0·137 g. (92 per cent) of crystalline azulene was obtained. After recrystallisation from alcohol, it formed blue leaflets, melting-point 98·5° to 99° C.

Other addition compounds can be split in a similar manner, for example that between guaiazulene and trinitrobenzene. Vetivazulene-picrate (0·577 g.) was similarly split by adsorption on a column (17 × 1·7 mm.) of alumina, which was developed with 40 ml. of a 1 : 1 mixture of cyclohexane and benzene and then washed with benzene. Picric acid remained on the column, whilst vetivazulene crystallised out from the filtrate after evaporation. A 95 per cent yield was obtained of material melting at 32° to 33° C.

Resin Pigments

Brockmann and Haase (1, 2) obtained the deep-red pigment dracorubin, $C_{32}H_{24}O_5$, from dragon's blood (*Sanguis draconis*), a resin prepared from an Indian palm, and used in varnish manufacture ; they used the following procedure.

One kilogram of the finely powdered material (Merck) was digested in 250-ml. portions with 1·5 litres of benzene and filtered after being allowed to cool ; the residue weighed 90 g. The filtrate was allowed to stand overnight after the addition of 10 g. of picric acid ; the brown picrate that formed was filtered off, thoroughly digested with benzene, and then twice shaken with benzene for 1 hour on a shaking machine. The residue was filtered off and dried in the air. The residue from 1 kg. of resin was suspended in 500 ml. of methyl alcohol, and the suspension was warmed on a water-bath and treated with a solution of 40 g. of potassium hydroxide in 100 ml. of water. After being warmed for a short time, the liberated dracorubin was filtered off and well washed with water, and the crystals were dried in a vacuum desiccator over calcium chloride, a yield of 36 g. of material, melting-point 287° C., being obtained.

This crude product was purified by chromatographing its chloroform solution on a column of aluminium hydroxide III. The lowest narrow brown zone of the chromatogram was washed out of the column on developing with chloroform, and the dracorubin was then eluted from the middle portion of the main bright-red zone with chloroform containing a little methyl alcohol. The solvent was evaporated off, and the crystalline residue was redissolved in chloroform and the solution was re-chromatographed. A chromatographically homogeneous product was thereby obtained in the form of garnet-red prisms of melting-point 315° C.

β-Hydrodracorubin prepared from dracorubin can also be purified by filtration through aluminium hydroxide III, using benzene containing 2 per cent of methyl alcohol for elution.

At the same time as Brockmann and Haase were carrying on their investigations, Hesse was also working on the pigments of dragon's blood. In his publications he refers to the principal pigment as "dracocarmin" ($C_{31}H_{26}O_5$) and to an accessory pigment as "dracorubin" ($C_{28}H_{24}O_7$), but in a later communication Hesse declares his intention of using in the future the older name dracorubin for the main pigment, thereby conforming to the nomenclature of Brockmann and Haase.

The powdered resin (580 g.) (Cæsar and Loretz) was exhaustively extracted with ether and the extract was precipitated by the addition of an equal volume of light petroleum. The deep-red precipitate was dissolved in 500 ml. of amyl alcohol and the solution was shaken alternately with N sodium hydroxide solution and water, until nothing further was extracted. The deep-red colour of the amyl alcoholic solution changed to brown when it was shaken with 2 N hydrochloric acid, after which it was concentrated under reduced pressure to about 200 ml. and the pigment hydrochloride (light brown, 15 g.) was precipitated with ether. The hydrochloride was dissolved in 200 ml. of chloroform and the solution was agitated with 2 N sodium hydroxide solution, whereupon the original deep-red colour returned. After being filtered from a small amount of residue, the solution was dried and chromatographed on a column (35 × 4·5 cm.) of Brockmann alumina.

Top : (brown)
deep red
red, containing the main pigment dracorubin (so-called " dracocarmin ")
(yellowish-red)
(pink)
Bottom : (brown)

The three lowest zones readily washed through the column on development with chloroform, when the receiver was changed and the main zone was then washed off the column. The solvent was distilled off from this fraction of the filtrate, and the resulting residue was warmed with a little chloroform or alcohol. Dracorubin crystallised out very readily in the form of shining crystals, melting-point 293° C., containing chloroform or alcohol of crystallisation respectively. The pigment formed an orange-yellow hydrochloride.

The column underwent no change on being further developed with chloroform, but on developing with a 1 : 1 mixture of chloroform and alcohol, a deep red band separated out from the very dark coloured zone and passed through into the filtrate. This fraction contained dracorubin (the " dracocarmin " of Brockmann and Haase); although it was very sparingly soluble, it could be crystallised from alcohol. It was best purified by converting it into the hydrochloride. Six different bands of various colours remained on the column, from which semi-crystalline pigments were obtained.

Colourless Constituents of Resins

Lariciresinol, $C_{20}H_{24}O_6$, was obtained in the following manner by Haworth and Kelly from the resin that exudes from recently felled larch trees (*Larix decidua*).

The fresh, viscous, cream-coloured resin (2·5 kg.) was twice extracted with 8 litres of boiling alcohol and the extract was distilled under reduced pressure. The brown viscous residue (1·5 kg.) was dissolved in 1 litre of hot alcohol and a solution of 900 g. of potassium hydroxide in 300 ml. of water was added. The potassium salt of lariciresinol separated from the solution on slow cooling, and after standing for 12 hours, it was collected and dissolved in water, and the solution was filtered. The filtrate was extracted with ether to remove impurities and then

acidified to Congo red with concentrated hydrochloric acid. The excess of acid was neutralised by the addition of sodium bicarbonate. A brown gum separated out on standing; after 12 hours the solution was filtered and the residue was freed from coloured impurities by repeated washing with warm sodium bicarbonate solution. The resulting brownish-yellow substance (270 g.) was dissolved in 1·5 litres of warm methyl alcohol and the solution was filtered through a column of alumina and then concentrated to about 400 ml. On standing for 12 hours, 170 g. of almost pure lariciresinol, melting-point 164° to 166° C., were obtained in the form of matted needles; after being twice recrystallised from methyl alcohol they had a melting-point of 167° to 168° C.

Fruit Pigments

Osajin

Walter, Wolfrom and Hess exhaustively extracted the chopped and dried (at 80° to 90° C.) fruits of *Maclura pomifera* Raf. (osage-orange) with hot light petroleum and extracted the osajin with ether. The ethereal solution was treated with charcoal, and filtered through a mixture of charcoal and fuller's earth. The pure crystalline pigment obtained on concentrating the filtrate could not be further purified by adsorption on alumina, followed by elution with glacial acetic acid. A yield of 93 g. of osajin of melting-point 182° to 186° C. was obtained from 1·6 kg. of dried fruits. It had the formula $C_{25}H_{24}O_5$ and had the characteristics of a phenol.

Rottlerin

According to Brockmann and Maier, the yellow pigment of the drug " kamala " (obtained from the glands of the epidermis of the fruits of *Rottlera tinctoria* Roxb.) has the formula $C_{30}H_{28}O_8$ and melting-point 201° to 202° C., which was not raised by fractional adsorption from benzene-petroleum solution on a column of calcium carbonate.

Iso-rottlerin, $C_{30}H_{28}O_8$

A solution of 4 g. of rottlerin in 500 ml. of alcohol was boiled under reflux for 96 hours, the colour of the solution becoming

paler. The solvent was distilled off and a 1 : 1 mixture of benzene and petroleum was twice distilled off from the residue, which was then dissolved in the mixture of benzene and petroleum ; the yellow solution was chromatographed on calcium carbonate (Merck). Two bands were formed, a brownish-yellow band at the top, and a pale yellow band at the bottom. On prolonged development with a 1 : 1 mixture of benzene and petroleum, the latter washed through ; the filtrate was distilled and the residue was crystallised, first from a mixture of benzene and methyl alcohol, and then from methyl alcohol. A yield of 1·6 g. of sulphur-yellow crystals of melting-point 180° C. (after being heated to 135° C. in vacuo) was obtained.

CHAPTER 4

APPLICATION TO SYNTHETIC DYESTUFFS

Ruggli and Jensen (1, 2) and Jensen investigated the adsorption behaviour of several different kinds of coal-tar dyes. Certain of the theoretical conclusions have already been discussed (p. 37).

(*a*) Basic Dyes

The order in which a number of basic dyes are adsorbed on activated alumina from aqueous solution is as follows:

Strongest	Victoria Blue B
↓	Methylene Blue D, Patent Phosphine G
	Crystal Violet 5BO, Fuchsine G, Safranine OO
	Brilliant Green, Malachite Green
Weakest	Auramine O

All the 36 possible binary mixtures of these 9 dyes were chromatographed, and a clean separation resulted with 32 of them, the method failing only with mixtures of Auramine and Malachite Green, Brilliant Green and Malachite Green, Patent Phosphine and Methylene Blue, and Fuchsine G and Safranine. In many instances the components of a ternary mixture were also separated from one another (see the coloured illustrations in the original publications). For example:

Top:	blue-black: Victoria Blue violet-blue: Methylene Blue	sharp narrow zones
Bottom:	broad yellow band, washed through into the filtrate: Auramine O	

A similar chromatogram was obtained when the Methylene Blue was replaced by Fuchsine.

(*b*) Acid Dyes

With a number of acid dyes the order of adsorption was as follows (the arrows indicate the direction of coupling):

Strongest
- Cloth Fast Black B (1-naphthylamine-5-sulphonic acid → α-naphthylamine → phenyl-peri-acid) and Cloth Fast Blue R (aniline → Cleve's acid → phenyl-peri-acid)
  (adsorbability of these dyes identical)
- Helvetia Blue (triphenylrosaniline-trisulphonic acid) and Orange II (sulphanilic acid → β-naphthol)
- Naphthol Yellow S (2 : 4-dinitro-α-naphthol-7-sulphonic acid)
- Erioglaucine supra (acid triphenylmethane dye) and Xylene Red B (sulphonated xanthene dye)

Weakest

(c) Substantive Dyes. Mono- and Disazo Dyes

DIAMINE ROSE FFB (dehydrothio-*p*-toluidine → 8-chloro-α-naphthol-3 : 6-disulphonic acid) : several narrow violet zones, then violet and pale violet bands, the latter easily washed through (" filtrate " [1]).

ERIKA G EXTRA (dehydrothio-*m*-xylidine → β-naphthol-disulphonic acid G) : broad light red zone, then blue and carmine-red bands, and violet " filtrate " with white regions below each band.

ERIKA B (dehydrothio-*m*-xylidine → α-naphthol-disulphonic acid) : dirty-yellow zone, then 3 filtrates : violet, pink and pink (for formula, see p. 40).

The examination of TECHNICAL CONGO RED affords an example of the chromatography of a commercial dye. Ruggli and Jensen (1) found that adsorption of the dye from pyridine solution gave rise to a chromatogram with the bands very close together, but that a much better spreading of the bands resulted when an aqueous solution was used. The order of the zones was the same in both instances.

One hundred millilitres of a 2 per cent solution were poured on to a column of alumina and the chromatogram was developed with 1·5 litres of water :

Top :	(*a*) a narrow, reddish-violet band
	(*b*) a broad, pink zone
	(*c*) an intense red main zone
Bottom :	(*d*) a broad, orange-yellow band

[1] In this section " filtrate " indicates that a constituent is so loosely adsorbed that it readily washes off the column, yet forms a temporary well-defined band. A rapid passage through the column is characteristic of such components.

The individual bands were separated ; each was eluted with boiling water and the eluates were evaporated. The isolation of the components was effected partly directly, and partly by dyeing mercerised cotton-wool and then recovering the dye with boiling water, evaporating the extract and crystallising the residue from methyl alcohol. When necessary these operations were repeated or the order was varied. Zone (*a*) gave a small amount of a dark red powder that dyed mercerised cotton-wool an intense reddish-brown. Zone (*b*) also yielded a dark red powder that dyed cotton red, the colour being sensitive to acid. Zone (*c*), the main zone, gave after three crystallisations a red powder that dyed cotton a pure red, sensitive to acid. Zone (*d*) gave a small amount of orange-red powder that dyed cotton yellowish-orange, the colour being stable to dilute acetic acid, but changed to reddish-violet by 5 per cent hydrochloric acid.

PURE CONGO RED, free from salt, obtained by crystallising three times from aqueous alcohol, was chromatographically homogeneous. In the presence of salt (2 g. to ½ g. of the dye in 100 ml. of water), the main zone that formed had the same appearance as usual but was rather narrower. It migrated down the column, leaving a second very narrow band at the top.

BENZOPURPURINE 4B (tolidine ⟶ 2 moles of naphthionic acid) was, like pure Congo Red, homogeneous on adsorption.

BENZOAZURINE G (dianisidine ⟶ 2 moles of 1-naphthol-4-sulphonic acid).

Top :	a dark blue band a broad, light blue zone	giving dyeings similar to those with the commercial product
	a greyish-blue filtrate, giving violet dyeings faster to ironing than those with the commercial product	
Bottom :	a red filtrate, giving brownish-violet dyeings	

The change in tint from bluish-violet to red is possibly due to polymerisation, as the same effect is obtained by ironing a dyed fabric, by warming a solution of the dye or by adding alcohol to its solution.

DIRECT SKY BLUE GREEN SHADE (dianisidine ⟶ 2 moles of 1-amino-8-naphthol-2 : 4-disulphonic acid) : 100 ml. of a 2 per cent solution were poured on a column (21 × 6·5 cm.) of alumina and developed with 1·5 litres of water :

Top : bluish-green band, giving pale greenish-blue dyeings
strong violet filtrate, dyeing bluish violet, not fast to ironing

Bottom : strong blue filtrate (main fraction) giving deep blue dyeings on cotton.

The following trisazo- and tetrakisazo-dyes gave complex chromatograms (the zones are given in order from top to bottom) :

DIANIL BLACK PR (*m*-phenylenediamine ← γ-acid ← benzidine-monosulphonic acid → γ-acid → *m*-phenylenediamine) : black, blue-black and white zones, then a dirty yellow filtrate, then a white zone and flesh-coloured and yellow bands (both the latter bands readily washed through).

CUPRANIL BROWN B (salicylic acid ← benzidine → γ acid → *m*-phenylenediamine) : chocolate brown, yellowish brown and white bands, then reddish-yellow filtrate, and white, violet, yellow, white, light yellowish-brown zones, easily washed through.

HESSIAN BROWN BBN (sulphanilic acid → resorcinol ← benzidine → resorcinol ← sulphanilic acid) : yellowish-brown, violet, white, reddish-yellow and yellow bands, then violet filtrate.

DIRECT BROWN J (*m*-aminobenzoic acid → *m*-phenylenediamine ← *m*-phenylenediamine → *m*-phenylenediamine ← *m*-aminobenzoic acid) : deep brown, yellowish brown, and pale brown zones, then reddish-brown filtrate, white zone, reddish-yellow filtrate, and pale yellow, canary yellow, and white zones, and brownish-yellow filtrate.

MIXTURES OF THE ABOVE DYES : As already mentioned on page 37, separation is in general easily effected when the number of azo-groups in the components is different. Thus, for instance, Congo Red and Diamine Rose FFB can readily be separated. The method fails, however, when the components are closely related, so that Erika B cannot be separated from Erika G extra, nor Congo Red from Benzopurpurine. Direct Sky Blue green shade is, however, adsorbed above Direct Blue 2B and can be separated from it (see formulæ on p. 40).

As is well known, the adsorption of Direct Blue 2B on cotton-wool is inhibited by the presence of salt, so Ruggli and Jensen (1) developed the column on which a mixture of Direct Sky Blue green shade and Direct Blue 2B had been adsorbed

with an 8 per cent solution of salt, in the expectation that the normal chromatogram would thereby be inverted. This, however, did not happen, but the two zones were brought closer together, so as to render the separation almost impossible.

For Sudan red, see page 50.

(*d*) Dyes of Pharmaceutical Importance

By adsorption on a column of alumina, Franck showed that the following commercial dyes were heterogeneous in character : Trypaflavin (3 : 6-diaminoacridine), Rivanol (2-ethoxy-6 : 9-diaminoacridine), Chrysarobin (3 - methyl - 1 : 8-dihydroxy-anthranol), Cignolin (1 : 8-dihydroxyanthranol) and Pellidol (diacetylamino-azotoluene).

CHAPTER 5

APPLICATION TO COLOURLESS AND FAINTLY COLOURED SUBSTANCES

1. Miscellaneous Aliphatic Compounds

(a) Removal of Traces of Dipalmityl ketone, $C_{15}H_{31}.CO.C_{15}H_{31}$, from Hentriacontane, $C_{31}H_{64}$ (Winterstein and Stein 1)

These two substances occur together in the unsaponifiable fractions of numerous plant-extracts and their separation is a matter of great difficulty except by chromatography. The experiment here described affords an illustration of the frequently observed phenomenon that substances judged by ordinary standards to be " chemically pure " can be still further purified by the adsorption method.

Hentriacontane (300 mg.), the melting-point and elementary analysis of which were identical with the accepted values, was dissolved in 100 ml. of petroleum and the solution was poured on to a column (6 × 1 cm.) of a mixture (5 : 1) of alumina and " fibrous alumina." On developing the column with 200 ml. of petroleum, the hentriacontane washed through into the filtrate. The adsorbent was allowed to stand for a short time with light petroleum containing a little methyl alcohol, this was filtered off, and the column was washed with the same solvent. The residue obtained by evaporation of the eluate consisted of dipalmityl ketone (2 mg.). The melting-point of the purified hentriacontane was distinctly sharper than that of the starting material.

(b) Isolation of an Aliphatic Hydrocarbon from a Plant

Simpson and Williams obtained a crystalline product (1·55 g.) of melting-point 70° to 75° C. from Mexican sarsaparilla

root after the removal of free acids and saponins. The solution of this material in 250 ml. of a mixture (2 : 2 : 1) of chloroform, light petroleum and benzene was poured on to a column of Brockmann alumina. About 90 per cent of the material crystallised out from the filtrate and had a composition corresponding with the formula $C_{23}H_{48}$ and melting-point 61° to 62° C. After being washed with 75 ml. of a mixture (1 : 1) of chloroform and benzene, the lower two-thirds of the column was eluted with a mixture of light petroleum and methyl alcohol. The eluate on evaporation gave a substance of the composition $C_{20}H_{42}O$ and melting-point 82° C. A third substance, melting-point 102° to 104° C., was obtained in small yield by elution of the upper third of the column.

(*c*) Fatty Acids and their Derivatives

Kondo has described the separation of free oleic acid from palmitic (or stearic) acid. A solution of 0·5 g. of the mixed acids in 40 ml. of a 1 : 1 mixture of benzene and petroleum was run on to a column (20 × 1·2 cm.) of alumina and the chromatogram was developed with 500 ml. of benzene. The oleic acid was adsorbed in the upper 6 cm. of the column and the palmitic acid in the lower 8 cm. ; the zone between contained a mixture of the two acids.

According to Wagner-Jauregg (2), the tribromanilide of tuberculic acid, $C_{28}H_{57}.COOH$, can be purified by filtering its benzene solution through Brockmann alumina and by several subsequent recrystallisations. It melts at 66° to 68° C.

Ralston, Harwood and Pool separated valeronitrile and lauronitrile from hydrocarbons on a column of silica gel, the nitriles being more strongly adsorbed from light petroleum than the hydrocarbons. The apparatus used is illustrated in Fig. 23, page 63.

(*d*) Separation of Different Sugars

Little work has so far been published on the chromatographic separation of the sugars. Hayashi described the separation of sucrose and dextrose from alcoholic solution by means of a column of blood-charcoal.

(*e*) Acetyl Cellulose

The work of Mark and Saito referred to below paves the way for the more complete investigation of highly polymerised substances by means of chromatography. The first of such experiments, carried out with technical cellulose acetate (Cellit), showed that substances of this type are split up into several components, distinguished from one another by their different viscosities. The acetyl contents of these different fractions were found to be the same, however, so that the separation depended on differences in the chain-length of individual polymers.

Plugs of glass-wool and asbestos were inserted into the end of a glass tube (42 × 3 cm.) and held in position by means of a cap of brass wire gauze. The tube was filled with three 10-g. lots of blood-charcoal (Kahlbaum), pieces of linen being interposed between sections of the adsorbent. The column was subjected to a pressure of about 1 atmosphere by means of compressed air ; 300 ml. of a 0·5 per cent acetone solution of Cellit were poured on to the column and compressed air was again applied. Charcoal adsorbs acetone with a noticeable evolution of heat ; when, however, the charcoal was first damped with acetone to overcome this difficulty, less Cellit was adsorbed, though the rate of filtration was about the same. The column was developed as long as acetyl cellulose continued to wash through into the filtrate, which was then concentrated.

The column was divided into three equal portions and each was eluted with 50 ml. of dioxan by shaking the adsorbent with the solvent and allowing to stand for a day. The troublesome filtration was done through a tube 1·5 cm. in diameter containing plugs of glass-wool and asbestos, and 7 g. of starch. The residue remaining after evaporating the eluate at 60° C. was dried in a vacuum desiccator over phosphorus pentoxide and blood-charcoal. The product was used for the determination of viscosity.

Table 18 shows the fractionation that resulted by this treatment ; the less viscous material was adsorbed in the uppermost third of the column, whilst the most viscous ran through into the filtrate.

TABLE 18

FRACTIONATION OF CELLIT AND RELATIVE VISCOSITIES OF THE FRACTIONS IN 0·1 PER CENT ACETONE SOLUTION

(η_r for acetone = 1·122) Mark and Saito

	Cellit "L 700" (η_r = 1·090)		Cellit "L 1000" (η_r = 1·163)	
	Contains Starting Material (%)	Viscosity	Contains Starting Material (%)	Viscosity
Top Zone . . .	30–35	1·049	30–35	1·101
Middle Zone . .	30–35	1·065	25–35	1·135
Lowest Zone . .	20–25	1·067	20–30	1·138
Filtrate . . .	5–10	1·097	10–15	1·198

Mark and Saito's results were confirmed for cellulose diacetate by Levi and Giera, whilst preparations of the triacetate showed some separation but no variation in viscosity.

2. TERPENES. ESSENTIAL OILS

(a) Separation of Geraniol, $C_{10}H_{17}OH$, and Limonene, $C_{10}H_{16}$ (Winterstein and Stein 1)

A solution of 4 g. of the mixture in 700 ml. of petroleum (boiling-point 70° C.) was chromatographed on a column (12 × 5·5 cm.) of activated alumina and developed with 300 ml. of petroleum. The column was eluted with light petroleum (boiling-point 30° to 50° C.) containing a little methyl alcohol. The eluate and the filtrate were each distilled, first through a Widmer column to remove the light petroleum, and then cautiously to 100° C. to remove the rest of the solvent. The residues were then distilled without attempting to fractionate them. In this way the filtrate yielded limonene and the eluate geraniol.

The separation of cineol, $C_{10}H_{18}O$, and dipentene, $C_{10}H_{16}$, was carried out in a similar manner.

(b) Aliphatic Halogenated Terpenes

Wagner-Jauregg and Arnold purified citronellyl chloride and citronellyl bromide by interposing a filtration through alumina

(Brockmann) between two distillations. When a light petroleum solution of the halide was run through a column of alumina, the adsorbent retained phosphorus-containing impurities formed during the synthesis. The procedure cannot be used for purifying geranyl bromide, as this reacts with alumina with evolution of heat.

(*c*) Cyclic Terpenes

Alumina was used by Huber for purifying a number of cyclic terpenes. The benzoate of 2-hydroxy-10-methyl-$\Delta^{1:9}$ octalin, $C_{11}H_{18}O$, was dissolved in a little benzene and the solution was added to a column (15 × 1·5 cm.) of alumina (Brockmann). On washing the column with benzene, the benzoate (1 g.) was found to be present in the first 60 ml. of the filtrate; a brownish-yellow zone was formed at the top of the column. The same method was used to purify the dinitrobenzoate and, among other substances, the benzoate of 9-hydroxy-13-methyl-$\Delta^{10:11}$-dodecahydrophenanthrene, $C_{15}H_{24}O$. Crude 9-keto-13-methyl-$\Delta^{10:11}$-dodecahydrophenanthrene, $C_{15}H_{22}O$, was also purified by adsorption on alumina, the compound being then washed off the column with benzene containing a little methyl alcohol.

Ruzicka and Sternbach purified tetrahydroxy-abietic acid-methyl ester, $C_{21}H_{36}O_6$, by filtering its benzene solution through a column of alumina. The same method of purification was applied to the tetramethyl ester of a tetracarboxylic acid, $C_{15}H_{22}O_8$, obtained by degradation of the above acid. The amount of adsorbent used was only five times the weight of the substance.

A mixture of β-amyrin, $C_{30}H_{50}O$, and erythro-diol, $C_{30}H_{50}O_2$, was separated (Ruzicka and Schellenberg) by chromatographing a solution in a 1 : 1 mixture of benzene and light petroleum (boiling-point 40° to 70° C.) on alumina (Brockmann). The β-amyrin passed through into the filtrate on washing with benzene and was twice recrystallised from alcohol; melting-point 198° to 199° C. The erythro-diol was washed off the column by developing with ether. When crude it had melting-point 230° C., and after purification melting-point 232° to 233° C.

Triterpenoids of Lichens

Asahina and Akagi extracted the Japanese lichen *Parmelia leucotyliza* Nyl. continuously with ether and, on allowing the extract to stand in a warm place, obtained crystals. A second crop of crystals was obtained on concentrating the filtrate; these were mixed with the first crop and the whole was fractionally crystallised from methyl alcohol. The most sparingly soluble fraction consisted of antranorin. The crystalline residue obtained by evaporation of the filtrate was stirred with cold 10 per cent potassium hydroxide solution, and the crystals were filtered off and recrystallised several times from methyl alcohol. A neutral fraction was thus obtained in the form of needles, melting-point 260° C., in a yield of 0·5 per cent. It was separated into two components by chromatography.

A solution of 2 g. in 1 litre of benzene was poured on to a column (25 × 1·8 cm.) of alumina (Brockmann), which was washed with benzene. The column was then cut into five equal parts and each was extracted with methyl alcohol. The topmost portion of the column yielded 0·5 g. of leucotylin, $C_{30}H_{52}O_3$, melting-point 330°, the next portion still more (0·6 g.) leucotylin, the third a mixture of leucotylin with zeorin, and the fourth 0·4 g. of zeorin, $C_{30}H_{52}O_2$, melting-point 250°. The mixture of the two compounds from the middle zone was separated by re-chromatographing. Since leucotylin is most probably a hydroxy-zeorin, it follows that the chromatographic separation of the two compounds depends on the differences in adsorption affinity due to the additional hydroxyl group in leucotylin.

(*d*) Essential Oils

Many oils contain in addition to stable components other substances such as geraniol, pinene, dipentene and limonene that are isomerised by adsorption on clays. In these instances, an evolution of heat occurs that is greater than that usually observed on adsorption, and this phenomenon has recently been investigated by Carlsohn and Müller (2). They have shown that the chromatographic separation, which can often be carried out with advantage, must be undertaken with natural bleaching-earths. Highly active artificial earths exhibit far too high

a catalytic activity, which may lead to structural changes of a fundamental character. This tendency to isomerisation is prevented by the presence of large amounts of strongly adsorbed substances such as alcohols, but the presence of ethyl or methyl alcohol or acetone does not affect it.

The catalytic effect of frankonite is greatest when the earth is first dehydrated at room-temperature and 1 mm. pressure over phosphorus pentoxide or sulphuric acid. The temperature effect, which runs parallel with isomerisation, can be measured by adding 1 ml. of the oil to 0·5 g. of frankonite in a jacketed test-tube and noting the maximum temperature attained. With the usual solvents the elevation of temperature was 3° to 12° C., but much higher values than this were observed with certain constituents of essential oils, a few of these being recorded in Table 19.

TABLE 19

RISE IN TEMPERATURE OF CONSTITUENTS OF ESSENTIAL OILS BY TREATMENT WITH FRANKONITE

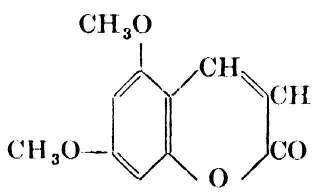
(Carlsohn and Müller 2)

Substance	Rise in Temperature	Remarks
Linalool	60°	Faintly coloured
Geranyl acetate	30°	Coloured
Linalyl acetate	76°	,,
Citronellal	72°	Faintly coloured
d-Limonene	114°	Brown, odour of cymene
l-Limonene	112°	,, ,, ,,
Dipentene	99°	Strongly coloured
Pinene	166°	Brown

Essential oils were split into their components by the following method. A mixture of 50 g. of floridin XXF (commercial grade, containing 17 per cent of water) and 150 g. of floridin XS were made into a column (12 × 8 cm.) and moistened with 500 ml. of petroleum (boiling-point 60° to 70° C.). A solution of 50 g. of the oil in 500 ml. of petroleum was then poured on to the column and the chromatogram was developed with 500 ml. of light petroleum (boiling-point 30° to 50° C.). As soon as essential oil began to make its appearance in the filtrate, as indicated by the odour, the first fraction of 250 ml.

was collected. Two or three further 250-ml. fractions were collected ; each was evaporated to remove the solvent and the residues were distilled under reduced pressure. The column was examined in daylight and in ultra-violet light, and any fluorescent portion was separated. The rest of the column was subjected to steam-distillation, thus liberating the strongly adsorbed components that serve in part as protective agents for the more labile compounds, as mentioned above.

BERGAMOT OIL : In a recent paper, Späth and Kainrath reported the isolation from Calabrian bergamot oil of a fraction boiling at 110° to 140° C., which could not be satisfactorily purified. It was accordingly chromatographed on a column of alumina from light petroleum (boiling-point 50° to 70° C.)



Limettin

solution and the chromatogram was developed with petroleum containing 1 per cent of methyl alcohol. On examining the column in ultra-violet light, four bands were seen. The lowest of these, which exhibited a blue fluorescence, was eluted with ether. The ethereal solution yielded crystals from which pure limettin, $C_{11}H_{10}O_4$, was ultimately obtained, melting-point 146° to 147° C.

3. Benzene and Naphthalene Series

(*a*) Phenols and Phenol Derivatives

According to Grassmann and Lang, ultra-chromatograms that are uniformly fluorescent throughout the column are given by the following phenols from methyl alcohol solution :

Phenol or catechol : faint violet colour on alumina or magnesia
Resorcinol : light bluish-violet on alumina or magnesia
Gallic acid : deep violet on alumina or magnesia
Phloroglucinol : yellowish on alumina, yellow on magnesia

For the use of ferric chloride as an aid to chromatographic analysis, see page 81.

DUROHYDROQUINONE-MONOCETYL ETHER, $C_{26}H_{46}O_2$ (John, Dietzel and Günther): An absolute alcoholic solution of durohydroquinone, $C_6(OH)_2(CH_3)_4$, was heated on the water-bath with an alcoholic solution of cetylbromide (1 mol.) and sodium hydroxide for 4 hours in a stream of nitrogen with efficient stirring. The solid material obtained on cooling the reaction mixture was washed with water and dissolved in acetone, a small amount of dicetyl ether remaining undissolved. The mono-ether crystallised out slowly from the acetone solution in the form of spherical nodules. The combined alcohol and acetone mother-liquors were treated with alkali to remove unchanged durohydroquinone and the residue was chromatographed on alumina from benzene solution. A further quantity of the di-ether was recovered from the filtrate, whilst the remainder of the mono-ether (melting-point 99·5° C.) stayed adsorbed on the column and was eluted from it by a mixture of alcohol and ether. A small amount of impurity was retained at the top of the column. The cetyl iodide formed by the action of hydriodic acid on the cetyl ether was purified by filtering a light petroleum solution through a short column of alumina.

SEPARATION OF PHENACETIN AND ACETANILIDE (Kondo): A solution containing 0·5 g. of a mixture of the two compounds in 100 g. of benzene was poured on to a column (4 × 1·8 cm.) of alumina and the chromatogram was developed with 200 ml. of benzene. The column was cut up into 5 portions and each was separately eluted with 20 ml. of a 7 : 3 mixture of ether and methyl alcohol. The residues remaining on evaporation of the solvent were crystallised from water. The fractions from the top two portions of the column consisted of pure phenacetin and that from the bottom portion of pure acetanilide.

(*b*) Nitranilines

SEPARATION OF *o*-, *m*-, AND *p*-NITRANILINE AND OF THE NITROPHENOLS (Karrer and Nielsen): A light petroleum solution of a mixture of the 3 nitranilines (0·15 g. of each) was poured on to a column of calcium hydroxide and the chromatogram was developed with light petroleum:

Top :	light yellow, containing *p*-nitraniline
	yellow : *m*-nitraniline
Bottom :	dark yellow to brownish : *o*-nitraniline.

The compounds were isolated by elution with benzene containing methyl alcohol, removal of the solvent and a second adsorption analysis of the residues.

In order to purify technical *o*-nitraniline or nitroxylidine (4 : 5-dimethyl-*o*-nitraniline) Kuhn and Ströbele first crystallised the material from benzene and then dissolved 50 g. in 300 ml. of benzene and filtered the hot solution through a column (5 × 5 cm.) of alumina. The column was surrounded by a hot-water jacket maintained at 70° C. to prevent the substance from crystallising out in the adsorption tube. Brown impurities remained adsorbed on the alumina, whilst the pure compound crystallised out from the filtrate.

The three nitrophenols were separated from one another in a similar manner by pouring a benzene solution on to a column of alumina or calcium carbonate ; the *p*-isomer was most strongly and the *o*-isomer least strongly adsorbed.

PREPARATION OF 3 : 5-DIMETHYL-6-NITROANILINE FROM 3 : 5-DIMETHYL ANILINE : Karrer and Strong (2) nitrated a solution of 8·3 g. of 3 : 5-dimethyl-aniline in 45 ml. of conc. sulphuric acid at 0° to 5° C. by means of a mixture of 6·64 g. of conc. nitric acid ($d = 1{\cdot}4$) and 7 ml. of conc. sulphuric acid. An orange-red precipitate was obtained when the reaction-mixture was poured on to ice. The suspension was made strongly alkaline with potassium hydroxide solution and extracted with ether. The extract was washed with alkali, dried, filtered and evaporated. The oily residue was extracted with two 400-ml. portions of boiling light petroleum.

The petroleum extract was chromatographed on calcium hydroxide and the column was developed with light petroleum until most of the colour had been washed through ; a narrow, yellow band remained at the top of the column. The residue left on distilling the filtrate was exhaustively extracted with several 100-ml. portions of light petroleum and the extract was filtered and concentrated. On cooling, yellow needles of slightly impure 3 : 5-dimethyl-6-nitroaniline, melting-point about 45° C., crystallised out. Yield, 4·8 g.

Kuhn, Desnuelle and Weygand added over a period of

5 minutes a mixture of 1·2 ml. of conc. nitric acid (d = 1·38) and 1·7 ml. of conc. sulphuric acid to a solution of 2 g. of the amine in 20 ml. of conc. sulphuric acid cooled to − 10° C. The resulting mixture was poured into ice-water, the suspension was filtered, and the residue, after being washed with water, was dried and dissolved in benzene. The solution was poured on to a column of alumina, and the substance, eluted with methyl alcohol and crystallised from dilute alcohol, had melting-point 53° C. A yield of 12 g. was obtained from 25 g. of xylidine.

(c) Intermediate Products in the Syntheses of Flavin

SCHIFF'S BASE FROM 3 : 5-DIMETHYL-6-NITROANILINE AND *l*-ARABINOSE (Karrer and Strong 2) : A mixture of 4 g. of the nitro-compound, 16 g. of the sugar, and 200 ml. of absolute methyl alcohol, was heated for 6 hours in a pressure flask at 105° to 115° C. After being allowed to stand, the contents of the flask were filtered from the unchanged arabinose that crystallised out on cooling, and the filtrate was chromatographed on alumina, the column being developed with absolute alcohol. Unchanged nitroaniline washed through into the filtrate, and a broad yellow zone formed on the adsorbent. This was eluted with hot 80 per cent methyl alcohol; after being concentrated, the eluate yielded crystals of the Schiff's base. This, recrystallised from alcohol, had melting-point 165° to 166° C. Yield, 1 g.

SCHIFF'S BASE FROM 4 : 5-DIMETHYL-2-NITROANILINE AND *l*-ARABINOSE : Kuhn, Reinemund, Weygand and Ströbele condensed pentoses with nitroxylidine (1 : 2-dimethyl-4-nitro-5-aminobenzene). A mixture of 0·5 g. of *l*-arabinose and 2·2 g. of nitroxylidine was heated for 6 hours at 110° to 120° C. with 30 ml. of absolute alcohol. The solution, after being allowed to cool, was drawn through a column of alumina and the chromatogram was developed with anhydrous alcohol. The unchanged nitroxylidine passed into the filtrate from which it readily crystallised out. The condensation-product formed an orange-yellow zone, which was eluted with 80 per cent methyl alcohol; the eluate was concentrated to one-fifth of its volume. The solid that separated was recrystallised from water, and formed orange-yellow prisms, melting-point

166° C. Yield, 0·58 g. Formula, $C_{13}H_{18}O_6N_2$. In this instance the unchanged starting-material was recovered quantitatively in a single chromatographic separation and was thus available for preparing a further batch of material.

1 : 2-DIMETHYL-4-NITRO-5-AMINOBENZENE-*d*-GLUCOSIDE: Kuhn and Dansi isolated the nitroxylidine-glucoside, resulting from the glucosidisation of nitroxylidine, by the following method: 1·35 g. of *p*-toluidine-glucoside and 4 g. of 1 : 2-dimethyl-4-nitro-5-aminobenzene were heated under reflux for 8 hours with 150 ml. of absolute alcohol and the orange-red solution was poured on to a column of alumina. The uppermost orange-red zone was eluted with a mixture of pyridine, methyl alcohol and water, the eluate was evaporated under reduced pressure, and the residue, crystallised from alcohol, had melting-point 213° C. (decomp.). Yield, 0·15 g. Formula, $C_{14}H_{20}O_7N_2$.

1 : 3-DIMETHYL-4-*d*-ARABITYLAMINO-5-NITROBENZENE, $C_{13}H_{20}O_6N_2$ (Kuhn, Desnuelle and Weygand): A mixture of 1·6 g. of *l*-arabinamine and 1 g. of 1 : 3-dimethyl-4 : 5-dinitrobenzene was heated in 10 ml. of 80 per cent alcohol at 135° C. for 10 hours. The contents of the flask solidified on cooling. The solvent was removed under reduced pressure, the residue was stirred with 10 ml. of water and 30 ml. of ether and the solid, filtered off and dried, had melting-point 130° C. The substance was purified by chromatographing its alcoholic solution on alumina, and eluting with a mixture of methyl alcohol, water, and pyridine. After recrystallisation from water it had melting-point 141° C. The preparation and purification of 1 : 3-dimethyl-4-*d*-ribitylamino-5-nitrobenzene, $C_{13}H_{20}O_6N_2$, was carried out in a similar manner.

l-ARABINOSE-2-NITRO-3 : 5-DIMETHYLANILIDE, $C_{13}H_{18}O_6N_2$ (Kuhn, Desnuelle and Weygand): A mixture of 4 g. of *l*-arabinose, 8 g. of 1 : 3-dimethyl-4-nitro-5-aminobenzene and 0·3 g. of ammonium chloride with 65 ml. of absolute alcohol was heated under reflux for 1 hour, the sugar dissolving after about half an hour. On chromatographing the reaction mixture on alumina, the excess of xylidine quickly ran through the column, and the bright yellow zone that formed was separated and eluted with a hot mixture of pyridine, methyl alcohol and water. The eluate was evaporated under reduced

pressure, and the final traces of pyridine were removed by repeated distillation with absolute alcohol. The residue, crystallised from water, had melting-point 171° C. Yield, 4·1 g.

2-NITRO-3-AMINO-5 : 6 : 7 : 8-TETRAHYDRONAPHTHALENE-N-*l*-ARABINOSIDE, $C_{15}H_{20}O_6N_2$ (Kuhn, Vetter and Rzeppa): A mixture of 3·8 g. of 2-nitro-3-amino-5 : 6 : 7 : 8-tetrahydronaphthalene, 3 g. of *l*-arabinose and 0·3 g. of ammonium chloride with 100 ml. of absolute alcohol was heated to boiling for 1¼ hours and the reddish-yellow solution was evaporated under reduced pressure. The residue was dissolved in 100 ml. of hot benzene and the solution was chromatographed on alumina. Unchanged nitro-amine ran through into the filtrate on developing the column with benzene, whilst the arabinoside was retained at the top of the column as an orange-yellow zone. This was eluted with a mixture (1 : 2 : 1) of pyridine, methyl alcohol and water, the eluate was evaporated under reduced pressure and the resulting syrup was crystallised from 50 ml. of alcohol. The product was thereby obtained in the form of golden-yellow needles (1 g.); its triacetate had melting-point 217° C.

d-ARABINOSE-2-NITRO-4 : 5-DIMETHYLANILIDE, $C_{13}H_{18}O_6N_2$ (Kuhn and Ströbele): A mixture of 5 g. of pure *d*-arabinose, 25 g. of 2-nitro-4 : 5-dimethylaniline and 0·5 g. of ammonium chloride with 120 ml. of absolute alcohol was heated on a boiling water-bath for 2 hours. Everything dissolved, and the red solution was diluted with 100 ml. of hot absolute alcohol and filtered hot through a column (30 × 5 cm.) of alumina jacketed with water at 75° C. Unchanged nitroxylidine ran through the column and crystallised out from the filtrate, whilst the arabinoside remained adsorbed. The column was washed until the filtrate was colourless, and the broad yellow zone was then eluted with a mixture (2 : 1 : 1) of pure methyl alcohol, pure pyridine and water. The eluate was evaporated under reduced pressure and the residue was freed from pyridine by distilling from it two 10-ml. portions of absolute alcohol. The product was finally crystallised from absolute alcohol. Yield, 8 g.

d-RIBOSE-2-NITRO-4 : 5-DIMETHYLANILIDE, $C_{13}H_{18}O_6N_2$: This was prepared in a similar way to the previous compound. For the adsorption, alumina was used at room temperature,

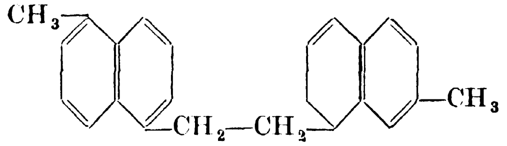
unchanged nitroxylidine being washed through on developing with benzene. Elution was carried out as described above.

d-GLUCOSE-2-NITRO-ANILIDE, $C_{12}H_{16}O_7N_2$: The reaction mixture was adsorbed from benzene solution on a column of alumina and the chromatogram was developed with benzene and eluted as described above.

TRITYL - *d* - MANNOSE - 2 - NITRO - 4 : 5 - DIMETHYLANILIDE, $C_{33}H_{34}O_7N_2$: The reaction-mixture was adsorbed from benzene solution on a column of alumina and the trityl compound was eluted with a mixture (1 : 1) of benzene and alcohol, leaving brown impurities behind on the column (Kuhn and Ströbele).

4. Di- and Triaryl-alkyls

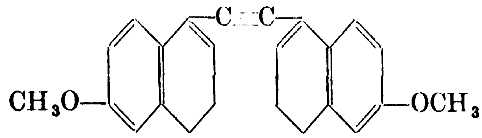
(*a*) TRIPHENYLMETHANE and TRIPHENYLCARBINOL can be separated by adsorption on alumina from light petroleum solution (Wieland, Ploetz and Indest). The hydrocarbon is but feebly adsorbed, whilst the carbinol forms a well-defined zone that fluoresces in ultra-violet light. The pure substance can be isolated from this zone by means of ether in an extraction apparatus. Tetraphenylmethane dissolved in a 1 : 1 mixture of benzene and light petroleum is not adsorbed on a column of alumina, and so can be separated from other substances that are adsorbed.



α-(7-methyl-naphthyl-1)-β-(5-methyl-naphthyl-1)-ethane

(*b*) α-(7-METHYL-NAPHTHYL-1)-β-(5-METHYL-NAPHTHYL-1)-ETHANE, $C_{24}H_{22}$ (Ruzicka and Hofmann): The crude product prepared by dehydrogenation had an ill-defined melting-point. It was dissolved in benzene and the solution was filtered through a column of alumina. The blue fluorescent compound that ran quickly through the column was collected; the yellow compound that passed through more slowly was rejected. The required compound formed needles, melting-point 74° to 75° C.

α-(5-Methyl-6-methoxy-naphthyl-1)-β-(7-methyl-naphthyl-1)-ethane, $C_{25}H_{24}O$, was purified in a similar manner.



Bis-6-methoxy-3 : 4-dihydro-naphthyl-1 : 1′-acetylene

(*c*) BIS-6-METHOXY-3:4-DIHYDRONAPHTHYL-1:1′-ACETYLENE was freed from a red impurity by chromatographing its solution in anisole on a column of alumina. The filtrate was light yellow, but became red again on standing. (Dane, Höss, Bindseil and Schmitt.)

5. Polycyclic Aromatic Hydrocarbons with Condensed Ring Systems and Related Substances

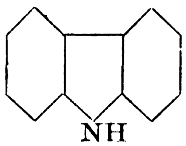
A. Chromatography of Naturally Occurring Hydrocarbons and Derivatives

The methods here given for the purification and separation of members of this class of compounds, which occur in coal-tar, are, unless otherwise stated, taken from the publications of Winterstein and Schön (2, 3), of Winterstein, Schön and Vetter, of Winterstein and Vetter and of Winterstein, Vetter and Schön. The relation between adsorption affinity and constitution has already been discussed on pages 33–37. The presence of impurities can be demonstrated in even the purest commercial preparations of these substances by means of the ultra-chromatogram.

Purification of Anthracene

(*a*) Anthracene (6 g.)–" for scientific purposes,"–Riedel was dissolved in petroleum and adsorbed on a column (25 × 6 cm.) of alumina. On development with petroleum, a brownish zone formed at the top of the column, and this did not yield crystals. On examining the column in ultra-violet light, a narrow band with a bright blue fluorescence was seen lower down the column, with a yellow band below it, and near the bottom a broad zone with a blue fluorescence. On further development, the bulk of the anthracene passed into the filtrate, the first runnings of which yielded a paraffin-like

substance. On concentrating the filtrate, anthracene crystallised out. The uppermost fluorescent zone was eluted with ether and the residue left on evaporation was sublimed in vacuo (0·5 mm.) at a temperature of 150° to 170° C. On adding petroleum to a benzene solution of the sublimate, colourless leaflets of carbazole were obtained in a yield of 350 mg., representing 5·8 per cent of the weight of starting material.



Carbazole

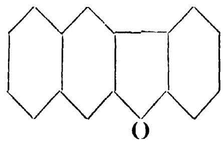
The purified anthracene gave an intense blue fluorescence, which was suppressed by the addition of 1/30,000 per cent of naphthacene.

(*b*) Anthracene (10 g.), Kahlbaum, was adsorbed on a column as described above. The column was developed until 6 g. of anthracene had run through into the filtrate. The whole of the column was then eluted with a mixture of petroleum and methyl alcohol, and the latter was removed from the eluate by washing it with water. The petroleum solution was dried and poured on to a second column of alumina. When half the anthracene (3 g.) had passed into the filtrate, this column also was eluted, and the material so recovered was chromatographed once more on a third column (8 × 4 cm.). This was developed with a large quantity of petroleum and was then examined in ultra-violet light. At the top of the column were two very narrow, bright yellow bands which were not investigated further; below these was a zone, with a bright blue fluorescence, from which pale yellow crystals were obtained on elution with ether. Sublimation in vacuo and recrystallisation from benzene yielded 5 mg. (0·05 per cent) of pure carbazole in the form of shining leaflets with a bright blue fluorescence. Below the carbazole zone was a narrow, yellow naphthacene band and below this a zone fluorescing dark blue, from which anthraquinone was obtained.

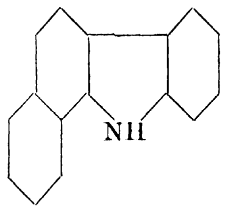
The original papers should be consulted for the isolation of naphthacene from anthracene oil. For the separation of anthracene and phenanthrene, see Ardaschew.

Purification of Chrysene (formula on p. 33) **and the Isolation of 1 : 2-Benzcarbazole**

Commercial chrysene (5 g.) was dissolved in 600 ml. of a mixture (10 : 1) of petroleum and benzene, and adsorbed on a column (14 × 4·5 cm.) of alumina, the chromatogram being developed with 500 ml. of petroleum. At the top of the column there formed a zone 1 cm. wide having a bright-blue fluorescence and below it a narrow yellow band. A third zone near the bottom of the column and the filtrate both exhibited a blue fluorescence. The first zone was eluted with ether, and the residue remaining after evaporation of the solvent was recrystallised from hexane and glacial acetic acid, giving 15 mg. (= 0·3 per cent) of 1 : 2-benzcarbazole. The middle zone contained naphthacene, whilst the bottom zone yielded completely colourless chrysene, which in spite of its apparent homogeneity contained traces of sulphur. On repeating the chromatography, the sulphur-compound was concentrated in the first 75 per cent of the filtrate, the remaining 25 per cent being sulphur-free. The sulphur compound therefore has practically the same adsorption-affinity as chrysene.



Brasan



1 : 2-Benzcarbazole

In a similar manner, brasan (2 : 3 : 5 : 6-dibenzo-coumarone) was isolated in a yield of 0·1 per cent from " pure " pyrene ; naphthacene and 2 : 3-(1′ : 2′-naphtho)-anthracene were also present (formulæ on pp. 33, 36). A thin layer of alumina-charcoal mixture above the alumina column is recommended in order to adsorb impurities more effectively.

Attempts to purify crude picene by chromatography were not very satisfactory.

Wagner-Jauregg was able to purify commercial acridine by running a benzene solution through alumina, the impurities remaining on the column and the acridine being recovered from

the filtrate. Whereas the original crude material had a pale blue fluorescence, the pure compound fluoresced yellow.

The foregoing examples illustrate the use of chromatography in purifying commercial preparations, whilst the following, also taken from the publications of Winterstein and co-workers, demonstrate the applicability of the method to the separation of artificial mixtures.

Separation of Naphthalene and Anthracene

A light petroleum solution (200 ml.) of 50 mg. of anthracene and 150 mg. of naphthalene was poured on to a column of alumina (150 g.) and the chromatogram was developed with 500 ml. of light petroleum. The anthracene was adsorbed on the upper half of the column, from which it was eluted with ether, the pure hydrocarbon being obtained on evaporation of the solvent. Pure naphthalene was obtained by distillation of the filtrate.

Separation of Anthracene and Chrysene (formula p. 33)

A petroleum solution, containing 50 mg. of the mixture, was adsorbed as above and developed until some of the material began to make its appearance in the filtrate. Two zones formed in the column, the upper of which, indicated by a dark-blue fluorescence in ultra-violet light, contained pure chrysene. The first portion of the filtrate contained pure anthracene.

Anthracene and pyrene could not be separated from one another.

Separation of Phenanthrene and Pyrene (formula p. 33)

The mixture was adsorbed according to the usual procedure and the filtrate was collected in four portions. The last of these contained pure pyrene.

Separation of Chrysene from Pyrene or from 1 : 2-Benzpyrene

This was accomplished as in the foregoing examples.

Benzpyrene Series

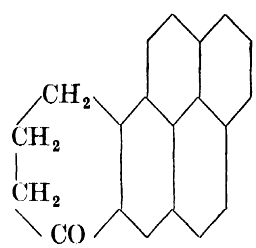
Separation of Perylene and 1 : 2-Benzpyrene (formulæ p. 36)

The mixture was adsorbed and developed as usual, when the column, on being examined in ultra-violet light, showed a broad yellow fluorescent band with another bright blue

fluorescent zone below it, one-fifth of the thickness of the former. The column was cut into five portions, the lowest of which yielded pure 1: 2-benzpyrene. The other portions gave mixtures, the two from the top having a preponderance of perylene and the two others containing an excess of benzpyrene.

The carcinogenic activity of 1 : 2-benzpyrene (also called 3 : 4-benzpyrene), discovered by Cook, Hewett and Hieger and confirmed by Schürch and Winterstein, renders the synthesis of this hydrocarbon of particular interest. It was purified by chromatographic analysis.

A mixture of 50 g. of 4′-keto-1′ : 2′ : 3′ : 4′-tetrahydro-1:2-benzpyrene (prepared chromatographically by Winterstein, Vetter



4′-keto-1′ : 2′ : 3′ : 4′-tetrahydro-1 : 2-benzpyrene

and Schön) and 30 g. of selenium was heated for 12 hours at 320° to 340° C. The liquid mass was poured into a mortar; when cool, the resin was finely powdered and extracted with 1 litre of boiling benzene. The extract was concentrated to a volume of 500 ml. and then poured on to a column of alumina 15 cm. high, covered with a 5-cm. layer of a 2 : 1 mixture of alumina and carboraffin. The chromatogram was developed with a mixture (5 : 1) of petroleum and benzene. The 1 : 2-benzpyrene quickly passed into the filtrate, but the development was continued until a green zone and then a brown zone washed out of the carbon layer into the alumina. The filtrate was concentrated to a small volume ; on the addition of light petroleum, greenish-yellow needles of 1 : 2-benzpyrene separated out, melting-point 177° (corr.). The yield was 22 g., equivalent to 47 per cent of the theoretical amount.

Derivatives of 1 : 2-Benzpyrene (formula p. 36 ; Windaus and Rennhak)

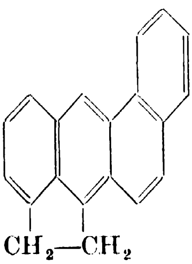
Mononitro-benzpyrene, $C_{20}H_{11}O_2N$. The crude material,

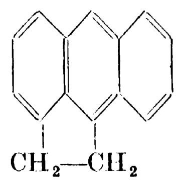
of melting-point 225° C., was adsorbed on a column (20 × 5 cm.) of ignited alumina from the least possible amount of benzene. Several dark red and greenish-black impurities were retained at the top of the column, whilst the main product, adsorbed at first as a broad, orange-yellow zone, was quickly washed into the filtrate on developing with benzene. The nitro-compound crystallised spontaneously from the first 750 ml. of filtrate, but the next 600 ml. had to be concentrated before crystallisation began. The product formed needles with a bronze lustre, melting-point 250° to 251° C. The washing was discontinued when the bands formed by impurities approached the bottom of the column. Acetylamino-benzpyrene, benzpyrene-mono-sulphonic acid methyl ester and benzpyrene-monocarboxylic acid methyl ester were purified in a similar manner. In the preparation of acetyl-benzpyrene, $C_{22}H_{14}O$, the product of the reaction of benzpyrene (1·26 g.) with acetic anhydride and aluminium chloride was extracted with benzene (100 ml.), and the extract was poured on to a column (20 × 3 cm.) of alumina. The chromatogram consisted of several greyish-brown bands at the top and a very broad greyish-brown zone below them. The column was washed first with high-boiling petroleum (about 600 ml.) until the filtrate was no longer coloured, then with a 1 : 5 mixture of benzene and petroleum, and finally with a 1 : 2 mixture of these solvents. The last filtrate contained the material from the broad zone ; on being concentrated it yielded orange-red needles of acetyl-benzpyrene, melting-point 186° to 186·5° C.

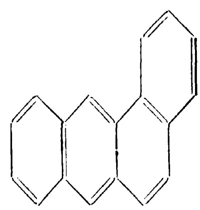
Derivatives of Aceanthrene, Benzanthracene, Benz-phenanthrene, Cholanthrene and Fluorene

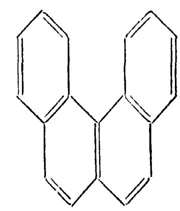
These have been purified by filtration through a column of alumina, usually in benzene solution, followed in many instances by conversion to the picrate (Fieser and Hershberg 1–5, Bruce and Fieser, Fieser and Riegel, Fieser and Seligman). Among the derivatives that have thus been purified are the following: 1 : 2-cyclopenteno-5 : 10-aceanthrene ; 1 : 2 - dimethyl-5 : 10-aceanthrene ; 1-methyl-5 : 6-cyclopenteno-anthracene ; 15 : 20-dimethyl-cholanthrene (twice chromatographed, then sub-limed) ; 1′ : 2′ : 3′ : 4′-tetrahydro-10-isopropyl-1 : 2-benzanthra-cene (melting-point after purification was 82° to 82·5° C.,

before purification 72·5° to 73·5° C.) ; 1′ : 2′ : 3′ : 4′-tetrahydro-1 : 2-benzanthracene ; 1′ : 2′ : 3 ′: 4′ : 9 : 10-hexahydro-1 : 2-benzanthracene ; 1 : 2 : 3 : 4-tetrahydro-10-methyl-1 : 2-benzanthracene ; 3-methoxy - 20 - methyl - cholanthrene ; 4 ′- bromo-7-methyl-8 : 9-dimethylene - 1 : 2-benzanthracene ; 1′-methyl-1 : 2 - benzanthracene ; 1′ - methyl - 1 : 2 - benzanthraquinone ;


Cholanthrene


Aceanthrene


1 : 2-Benzanthracene


3 : 4-Benzphenanthrene

1′ : 10-dimethyl-1 : 2-benzanthracene. The original paper by Fieser and Hershberg (4) should be consulted for details of the purification of 10-alkyl-1 : 2-benzanthracenes and 10-methoxy-1 : 2-benzanthracene. Other compounds belonging to this group that have been purified in a similar way are: 7-cyano-10-methyl-1 : 2-benzanthracene (Newman and Orchin), 3 : 4-benzphenanthrene and 2 : 9-diethyl-3 : 4-benzphenanthrene (Newman and Joshel). The synthesis of 9 : 10-dialkyl-benzanthracenes was described by Newman and by Bachmann and Chemerda.

Separation of two Dinitro - methylcholanthrenes, $C_{21}H_{14}(NO_2)_2$ (Rossner)

The crystalline nitration product of methyl cholanthrene was adsorbed from benzene on active alumina and the column was developed with a 1 : 1 mixture of benzene and light petroleum. Two zones were formed ; the upper, dark red in colour, contained the isomer of melting-point 257° C., and the lower, orange-coloured, the isomer of melting-point 224° C. Methyl cholanthrene itself was purified in the same way.

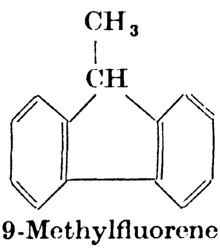
Amino-cholanthrene, $C_{21}H_{17}N$

The phenylhydrazone, $C_{33}H_{49}N$, of cholestan-3-one was dehydrogenated with selenium, first for a short time at 160° C., then for 16 hours at 320° C., and finally for 30 hours at 340° C.

The reaction-product was extracted with ether, and the residue left after removal of the solvent was sublimed in a high vacuum. At 180° to 200° C., a fraction was obtained that would not crystallise and was readily oxidised on exposure to air. It had therefore to be kept in an atmosphere of carbon dioxide. Adsorption on alumina from a 1 : 1 mixture of benzene and light petroleum resulted in 10 fractions, of which the middle one, deep yellow in colour and strongly fluorescent, after being sublimed in high vacuum, gave crystals, melting-point 225° C., from benzene. Rossner treated other crude dehydrogenation products in a similar manner.

Separation of an Isomorphous Crystalline Mixture of Fluorene and 9-Methylfluorene (Wieland and Probst).

The mixture had every appearance of being a single substance ; amongst other things, the melting-point of the dibromo-compound was constant. Its composite nature was only apparent when chromatographed on a column (100 × 1 cm.) of alumina, fluorene being more strongly adsorbed than its methyl derivative. The chromatographing was repeated several times and the fluorene ultimately obtained hardly fluoresced at all in ultra-violet light, so that its name is evidently misleading !



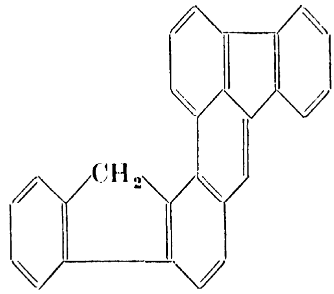
9-Methylfluorene

Hydrocarbon, $C_{27}H_{16}$

This compound was obtained by Wieland and Probst in an attempted thermal decomposition of polymerised diphenylene-ethylene in high vacuum. On chromatographing a benzene solution of the product on alumina, dark-coloured impurities were retained in the upper portion of the column, whilst the bright yellow hydrocarbon readily washed through. The compound was ultimately obtained pure by alternate crystal-

lisation and adsorption analysis, using chloroform as solvent It formed bright yellow leaflets, melting-point 198° to 199° C.

According to Kuhn, Vetter and Desnuelle, 2-nitro-3-amino-hydrindene can be purified by filtering the benzene solution through a column of alumina.

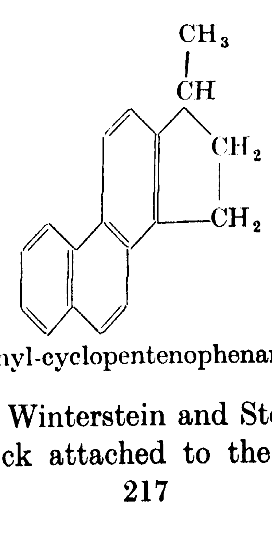


Hydrocarbon, $C_{27}H_{16}$ (presumed formula)

B. Chromatography of Dehydrogenation Products of Natural Substances

(a) Purification of a Hydrocarbon, $C_{18}H_{16}$ (γ-methyl-cyclopenteno-phenanthrene) obtained from Cholesterol (Diels and Rickert)

The product obtained by selenium dehydrogenation of cholesteryl chloride formed beautiful crystals from alcoholic solution, but, in spite of its appearance, it was not homogeneous, for adsorption on alumina separated from it both solid and oily impurities. Strangely enough, the melting-point was not perceptibly altered by this treatment.



γ-Methyl-cyclopentenophenanthrene

The apparatus of Winterstein and Stein (Fig. 9, p. 59) was used with a stop-cock attached to the adapter. Petroleum

(boiling-point 70° to 80° C.) was employed both as solvent and for developing the column. A solution of about 3 g. of the apparently pure substance in 95 ml. of solvent at 15° to 20° C. was drawn slowly through a column (20 × 2 cm.) of alumina by gentle suction. A yellow band containing an oily impurity travelled slowly down the column and by the end of the experiment had moved 8 to 10 cm. After all the solution had been added from the dropping-funnel, 3 to 4 ml. of petroleum were transferred to it to wash out the remainder of the substance and to develop the column. The stop-cock on the adapter was now closed and the receiver was changed.

As soon as hydrocarbon made its appearance in the filtrate, as indicated by a change in the optical properties of the liquid and the appearance of a blue fluorescence in ultra-violet light, 10 drops were collected and the receiver was again changed. The fraction that was next collected contained the main product and the filtration was continued until the dropping-funnel was empty and no more liquid could be sucked through the column. The strongly fluorescent solution was concentrated by heating under reduced pressure and the adsorption was repeated in a smaller column. In this second chromatogram a much smaller amount of the yellow impurity was adsorbed, and the band corresponding to it moved at the most only 2 cm. down the column. A yield of 1·9 g. of γ-methylcyclopenteno-phenanthrene was obtained in the form of white leaflets having a bright blue fluorescence ; melting-point 124° to 125° C.

At the end of the process the column was examined in ultra-violet light. The region containing the yellow band exhibited a strong bright green fluorescence, whilst the rest of the tube showed the blue fluorescence characteristic of the main product. Two crystalline impurities, both hydrocarbons, were isolated subsequently.

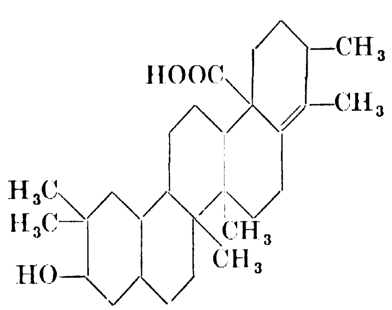
(*b*) Hydrocarbon, $C_{26}H_{26(24)}$, obtained by Dehydrogenation of Ergosterol

On oxidation with chromic acid the hydrocarbon gave a mixture of a bright yellow ketone, $C_{26}H_{24}O$, and a red quinone, which could not be separated from one another by crystallisation. Ruzicka and Goldberg were able to separate them by pouring a benzene solution of the mixture on to a column of

alumina, the quinone being adsorbed and the ketone passing into the filtrate, from which it was recovered by evaporation of the solvent. After several recrystallisations it melted at 175° to 175·5° C. Another hydrocarbon, $C_{27}H_{28(26)}$, was investigated by the same method.

(c) Hydrocarbons obtained by Selenium Dehydrogenation of Quinovic and Pyroquinovic Acids

By the dehydrogenation of quinovic acid, the aglycone of quinovin, the bitter principle of cinchona bark, and of pyroquinovic acid, Wieland, Hartmann and Dietrich obtained the



Pyroquinovic Acid

hydrocarbons C_nH_n and $C_{25}H_{20}$ respectively. These could only be purified satisfactorily by chromatography. A benzene solution (25 ml. containing 150 mg. of substance) was filtered through a column (15 × 1 cm.) of activated alumina, and the hydrocarbon $C_{25}H_{20}$ that passed through was converted into a quinone. A suspension of 0·25 g. of the hydrocarbon in 50 ml. of glacial acetic acid was heated under reflux for 3 hours with 0·2 g. of chromic oxide and the liquid was filtered hot to remove a small amount of insoluble residue. The oxidation-product was diluted with water and the brick-red quinone that precipitated was filtered off, washed with water, and dried. It showed no tendency to crystallise and was therefore purified by adsorption.

A solution of 200 mg. of the crude quinone in 120 ml. of benzene was poured on to a 15-cm. column of alumina. Unchanged hydrocarbon, melting-point 295° C., separated out at once from the colourless filtrate, and more was recovered in the filtrate on developing the column with 150 to 200 ml. of benzene. A red band 4 cm. wide, due to the quinone, remained on the column, which was extracted with benzene. The

solution on standing deposited shining red leaflets, which, after recrystallisation, first from benzene and then from pyridine, had melting-point 252° to 255° C.

6. Sterols and Bile Acids

Chromatography can be of great value in the investigation of sterols, and if only this technique had been applied earlier in this field, much of the confusion that exists in the literature would have been avoided ; for many substances, once believed to be individual sterols, and as a result given specific names, would have been shown to be mixtures of two or more sterols already known.

Plant Sterols

Sitosterol from the Pollen of *Typha* species

Kuwada and Morimoto adsorbed the crude sterol from light petroleum solution on a column of alumina and thereby showed that the so-called "α-typhasterol" was simply sitosterol, $C_{29}H_{50}O$. The filtrate from the chromatogram was evaporated and the residue was dissolved in acetone ; the solution yielded shining platelets of pentacosane, $C_{25}H_{52}$.

Minor Sterols of Yeast

Cryptosterol, $C_{30}H_{49}OH$, is found in the dark viscous residue left in the mother-liquors after crystallisation of the other sterols from the unsaponifiable fraction of yeast fat. Wieland, Pasedach and Ballauf filtered a solution of 500 g. of this viscous material in 1 litre of benzene through a 1·2 m. column of alumina (1·5 kg.) and washed the column with benzene. The first portion of the filtrate gave on evaporation an oily residue insoluble in methyl alcohol, whilst the second fraction gave an oil soluble in methyl alcohol, the solution on standing yielding crystalline cryptosterol, melting-point 134° to 136° C. The third portion of the filtrate, which was bright yellow in colour, gave a mixture of sterols having a much lower melting-point. The crude cryptosterol was dissolved in the least possible amount of benzene and adsorbed on ten times its weight of alumina. Some of the cryptosterol was recovered from the filtrate, but most of it was recovered from the column by extraction with hot ether. A yield of 25 g. of the pure sterol

was obtained, melting-point 138° to 140° C.; material prepared without using chromatography invariably had a lower melting-point than this. The lower layer of the main chromatogram gave on elution a mixture of sterols, the middle layer a mixture of sterols in which ergosterol predominated, and the upper two-fifths of the column non-sterol compounds.

Cryptosterol acetate can be purified in the same way, but is less strongly adsorbed than the free sterol. Cryptostenone, $C_{30}H_{50}O$, the oxidation product of dihydrocryptosterol, and chloro-cryptostenone, $C_{30}H_{49}OCl$, can be purified in a similar manner.

Cryptostadienone, a doubly unsaturated ketone, $C_{30}H_{48}O$, was obtained from cryptosterol by the following method. A solution of 1·05 g. of the sterol in 30 ml. of warm acetic acid was treated with 0·6 g. of chromic acid (dissolved in a little water), the mixture was heated at 70° to 80° C. for 3 minutes and then poured into water. The aqueous suspension was extracted with ether, and the ethereal extract, after being shaken with aqueous alkali solution, was dried and distilled. The oily residue (860 mg.) could not be induced to crystallise. A solution of 1·9 g. in 20 ml. of benzene was therefore poured on to a column (40 × 0·7 cm.) of alumina. The first runnings left no residue on evaporation, but the filtrate obtained on washing the column with 10 ml. of benzene yielded 410 mg. of solid. Impurities remained as yellow ill-defined bands at the top of the column. The washing was continued with further 10-ml. quantities of benzene until no more material was eluted. A residue weighing 2 mg. was obtained from the tenth filtrate. The eluted material was combined (800 mg.) and dissolved in a mixture of acetone and methyl alcohol, from which it eventually crystallised. After three recrystallisations it had melting-point 65·5° to 67° C.

The purification of two other sterols of yeast, the isomeric zymosterol and ascosterol, $C_{27}H_{43}OH$, can be accomplished by adsorption on alumina (Wieland and Kanaoka), though they were actually isolated by a different method.

Tritisterols

These were discovered by Karrer and Salomon (1) in wheat germ oil (*Triticum*) and form a special class of sterols character-

ised, amongst other things, by their surprisingly high solubility and by the amorphous nature of their digitonides. The initial stage of the isolation of the tritisterols was a chromatographic separation, identical in principle with that previously used by Drummond, Singer and MacWalter. This was followed by a separation of the digitonides, but only the chromatographic separation will be described here.

A solution of 75 to 80 g. of the unsaponifiable matter in 3·0 to 3·5 litres of methyl alcohol was heated until only a small amount of material remained undissolved. On being allowed to stand overnight at — 10° to — 15° C., sterols crystallised out from the reddish-yellow solution and were filtered off and washed until free from colour. The filtrate was concentrated under reduced pressure until it began to go turbid, when it was transferred to a conical flask and diluted to 250 to 300 ml. with methyl alcohol. The solution was allowed to stand overnight in the cold room, when a red semi-solid layer separated at the bottom of the flask and sterol crystals were deposited on the walls. These were separated by rapid filtration of the liquid, and the oily layer was digested several times with methyl alcohol, the extracts being used for washing the sterols. The combined filtrate and washings were evaporated under reduced pressure and the dark oily residue so obtained was dissolved in light petroleum.

After being twice washed with water, the petroleum solution was dried and poured on to a column (70 × 3·5 cm.) of alumina, and the chromatogram was developed by washing with large volumes of petroleum (boiling-point 60° to 90° C.). The filtrate first obtained contained a colourless " Fraction A," followed by a filtrate containing a yellow substance eluted from the column. The washing was continued until this was replaced by a second colourless " Fraction B." (For the working up of this fraction, see p. 266.) The column then had the following appearance (the thickness of each layer is given in millimetres) :

Top :	20–30	greenish-yellow
	100	sand-coloured
	150–180	reddish-brown at the top, changing gradually into dark yellow at the bottom (" Zone D ")
Bottom :	350–400	sand-coloured : main zone, the " Fraction C " of Drummond, Singer and MacWalter

The tritisterols were present in " Fraction C " and in the lower part of " Zone D " from which they were eluted with a mixture (4 : 1) of methyl alcohol and ether. After removal of the solvent, " Fraction C " gave a reddish-brown oil amounting to 25 per cent of the starting material. When 25 g. of this material had accumulated, it was dissolved in light petroleum and re-chromatographed :

Top :	10–20	deep yellow (less than 1 g.)
	150	brownish-yellow, " Fraction D " (5 g.)
Bottom :	500	sand-coloured, " Fraction C " (15 g.)

" Fraction C " contained only traces of " normal " sterols and was fractionated by a novel type of digitonin treatment, for a description of which the original paper should be consulted.

Sterols from Rice-germ Oil

Todd, Bergel, Waldmann and Work investigated wheat-germ oil and rice-germ oil by a method similar to that used by Karrer and Salomon (1). From the latter material the three isomeric α-, β- and γ-orysterols, $C_{30}H_{50}O$, which closely resemble the tritisterols, were isolated ; β-orysterol is possibly identical with α-tritisterol.

Rice-germ oil (2 kg.) was saponified by heating with 15 per cent methyl alcoholic potassium hydroxide solution for 5 hours, and the fatty acids were precipitated as barium salts. The unsaponifiable matter (37·9 g.) was extracted with ether, and the bulk of the sterols (22·7 g.) were removed by allowing the methyl alcoholic solution to stand at 0° C. The remainder of the sterols (2 g.) was removed by treatment with digitonin, leaving 13·2 g. of sterol-free oil. Eleven grams of this oil were fractionally distilled at 0·1 to 0·2 mm. pressure, and the portion (7·1 g.) distilling at 190° to 275° C. was dissolved in 750 ml. of light petroleum (boiling-point 40° to 60° C.) ; the solution was poured on to a column (31 × 3·5 cm.) of alumina, which was developed with light petroleum containing a trace of benzene. The following chromatogram was produced :

Top :	23	yellowish-brown (0·22 g.)
	110	greenish fluorescence in ultra-violet light (3·74 g.)
	55	bright blue fluorescence (0·6 g.)
Bottom :	115	blue fluorescence (0·33 g.)

The main green fluorescent zone was eluted with a mixture

(4 : 1) of methyl alcohol and peroxide-free ether ; the eluate was distilled and the residue was esterified by heating it with *p*-nitrobenzoyl chloride (6 g.) and pyridine for 2 hours. After being allowed to stand overnight, the product was poured on to ice and the mixture was acidified with sulphuric acid. The sticky precipitate was dried and extracted, first with 500 ml. of boiling light petroleum (boiling-point 40° to 60° C.) and then with several portions of peroxide-free ether.

The ethereal extract was shaken with alkali solution to remove *p*-nitrobenzoic acid, and the residual ester was freed from solvent and crystallised several times from acetone. It formed colourless needles, melting-point 227° to 228° C., which were hydrolysed by heating for 2 hours with butyl alcoholic potassium hydroxide solution. The alcohol was removed by steam-distillation, and the flocculent precipitate that resulted was extracted with ether ; the β-orysterol so obtained was crystallised from a mixture (5 : 1 : 4) of alcohol, water and acetic acid. It had melting-point 113° to 114° C.

The light petroleum extract was divided into a gelatinous, readily soluble portion and a sparingly soluble portion. The former (1·13 g.) was chromatographed on alumina from solution in a mixture (9 : 1) of light petroleum and benzene, and the column was developed with 1 litre of the same solvent mixture. The middle section of the column, characterised by strong ultra-violet absorption, was eluted with peroxide-free ether, giving 0·95 g. of a yellow semi-crystalline ester. This was hydrolysed by heating for 2 hours with hot 10 per cent methyl alcoholic potassium hydroxide solution. The α-orysterol so obtained was crystallised by slow concentration of its alcoholic solution over phosphorus pentoxide, the pure substance melting at 121° to 122° C.

Animal Sterols

γ-Sitosterol, $C_{29}H_{50}O$, from the toxic secretion of the toad (*Bufo vulgaris*)

Hüttel and Behringer evaporated the light petroleum extract of the poison and repeatedly extracted the residue with boiling methyl alcohol. On cooling the extract, crude sterol precipitated and this was chromatographed from benzene

solution on alumina. The column was divided up according to the results obtained with the Liebermann reaction, and according to the fluorescence observed in ultra-violet light. The sitosterol was present in the main zone, and was purified by repeated chromatography. A yield of 2·5 g. was obtained from the secretions of 33,000 toads.

Lanosterol: According to Dorée and Petrow, lanosterol, $C_{30}H_{50}O$, from wool fat can be purified by chromatography.

Cholestadienol, $C_{27}H_{42}O$, prepared by Dane and Wang from cholesterol dibromide, was separated into two components by chromatography on alumina. Though both components possessed conjugated double-bonds, their absorption spectra were different. Further details are promised in a subsequent publication.

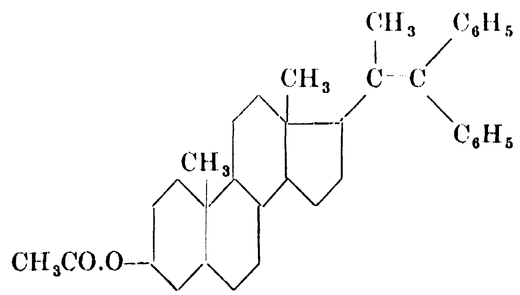
2 : 4-Dibromocholestenone, $C_{27}H_{40}OBr_2$, was subjected to a final stage of purification by Dane, Wang and Schulte by chromatographing on alumina, whereby the melting-point was raised from 191° to 203° C.

Iso-ergosterone, $C_{28}H_{42}O$, an isomer of cholestadienone, was obtained by Wetter and Dimroth from the mother-liquors of ergosterone by chromatography on alumina from light petroleum solution.

For dehydrogenation products of sterols, see page 217 *et seq.*

Bile Acids

(*a*) 1 : 1-Diphenyl-methyl-(3-acetoxy-ætiocholyl-)-ethylene, $C_{36}H_{46}O_2$, a by-product obtained by Sawlewicz and Reichstein during the conversion of lithocholic acid into ætio-lithocholic acid, could not at first be obtained crystalline. Accordingly, 1 g. was dissolved in 200 ml. of pentane and the solution was



1 : 1-Diphenyl-methyl-(3-acetoxy-ætiocholyl)-ethylene

filtered through a column of alumina (20 g.) prepared with pentane. The column was washed with several 150-ml. portions of the same solvent, the filtrate from each being separately distilled and tested. The first filtrate and the first wash-liquor contained only a little diphenyl; the succeeding seven solutions gave altogether 0·5 g. of a resinous, almost colourless solid, which slowly crystallised after the addition of a few drops of glacial acetic acid. After being washed with a little acetic acid, 0·32 g. of the compound, melting-point 161° to 163° C. (sintered at 157° C.), was obtained.

(*b*) The isomeric cholenic acids obtained by removal of water from 6-hydroxy-allocholanic acid can be separated at least partially from one another by chromatographing the alcoholic solution on alumina (Wieland, Kraus, Keller and Ottawa). Examination by ultra-violet light indicated the presence of four indistinct zones, in which the proportion of the two isomers varied progressively from the top of the column to the bottom.

7. Sapogenins

(*a*) **Purification of Crude Sarsasapogenin** (from *Radix Sarsaparillæ*)

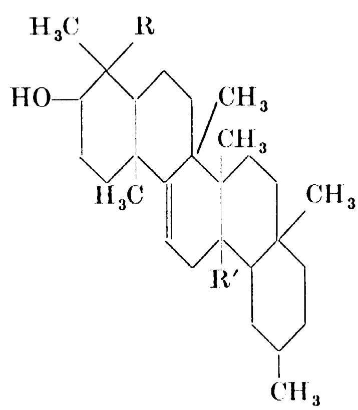
This was most readily effected by filtering a 4 to 5 per cent solution in benzene through a column of alumina (Askew, Farmer and Kon). Impurities were retained on the column, whilst the filtrate on evaporation gave a residue that crystallised from acetone. The product had melting-point 197° to 198° C.; $[\alpha]_D = -75°$. Fieser and Jacobsen used a similar technique.

(*b*) **Isolation of Soya-bean Sapogenols A, B, C and D** (Ochiai, Tsuda and Kitagawa 1, 2)

The crystallised saponin (100 g.) was heated under reflux for 30 hours with a mixture of conc. hydrochloric acid (200 ml.) and methyl alcohol (1·2 litres); the liquid was concentrated under reduced pressure and then poured into water and the resulting suspension was filtered. The precipitate was extracted with ether, the ethereal solution was evaporated, and the residue was dissolved in 2 litres of benzene. On being allowed to stand overnight, this solution deposited crystals

(20 g.), melting-point 248° to 252° C., of crude sapogenol B. The filtrate was poured on to a column (60 × 3 cm.) of alumina, which was developed with 1 litre of benzene, and then divided into 4 parts, each of which was separately eluted with methyl alcohol. Zone I from the top of the column gave 1·5 g. of crude sapogenol A of melting-point 265° to 280° C. Zones II and III gave crystals which were treated with hot methyl alcohol, the more soluble fraction yielding 8 g. of crude sapogenol C, melting-point 233° to 235° C., and the less soluble 0·5 g. of sapogenol D, melting-point 295° to 298° C.

SOYA-SAPOGENOL A, $C_{30}H_{50}O_4$: The crude material (1·5 g.) was dissolved in 500 ml. of benzene and the solution was run through a column (30 × 1·5 cm.) of alumina, which was



Skeleton of the sapogenols (Ruzicka)

developed with 200 ml. of benzene. The top quarter of the column was eluted with methyl alcohol, and the eluted material was recrystallised twice from methyl alcohol. The pure compound formed dextro-rotatory plates, melting-point 308° to 312° C.

SOYA-SAPOGENOL B, $C_{30}H_{50}O_3$: The crude material (20 g.) was several times recrystallised from methyl alcohol and was then dissolved in 2 litres of benzene ; the solution was filtered through a column (28 × 1·7 cm.) of alumina. One-seventh of the column was removed from the top and a similar quantity from the bottom, the rest was eluted as in the previous instance, and the product was recrystallised from methyl alcohol. It formed needles, melting-point 258° to 259° C., which were dextro-rotatory.

SOYA-SAPOGENOL C, *$C_{30}H_{50}O_2$: One gram of the crude* material was chromatographed on a column (25 × 1 cm.) of alumina from 100 ml. of benzene, the column being developed with 100 ml. of a mixture (1 : 1) of benzene and chloroform. The filtrate was distilled and the residue was dissolved in methyl alcohol. On concentrating the solution, needle-shaped crystals separated out which, after two recrystallisations, had melting-point 238° to 239° C.; they were dextro-rotatory.

SOYA-SAPOGENOL D, $C_{30}H_{50}O_3$: The crude material was crystallised from acetone-methyl alcohol mixture, giving lævo-rotatory granular prisms, melting-point 298° to 299° C.

(*c*) Derivatives and Degradation Products of Soya-sapogenol B (Ochiai, Tsuda and Kitagawa 1, 2; Tsuda and Kitagawa)

The acetate of soya-sapogenol B was purified by filtering the benzene solution twice through activated alumina. The product, $C_{29}H_{44}O_2$, obtained by chromic acid oxidation was similarly purified; evaporation of the first portion of the filtrate, followed by crystallisation of the residue from acetone, gave prisms of melting-point 254° to 256° C. The hydrocarbon, $C_{13}H_{14}$, obtained by selenium dehydrogenation of the sapogenol yielded a crystalline picrate, which was decomposed by running a 1 per cent solution in benzene through a column of alumina. The purified hydrocarbon was then isolated as the styphnate.

Details concerning the chromatography of degradation products of sarsasapogenin and smilagenin are given by Farmer and Kon.

(*d*) Separation of Oleanol, $C_{29}H_{47}OH$, and Oleanylene, $C_{29}H_{46}$ (Winterstein and Stein 1)

The product of decarboxylation of oleanolic acid was dissolved in petroleum and the solution was freed from acid by washing three times with saturated barium hydroxide solution. The dried petroleum solution was drawn through a column (14 × 4·5 cm.) of activated alumina, which was then washed with 400 ml. of petroleum. The filtrate was evaporated, and the residue on being crystallised from acetone yielded 2·5 g. of oleanylene. The adsorbed oleanol was eluted from the column

with a mixture of ether and methyl alcohol, the solution was evaporated and the residue was crystallised from acetone. A yield of 2·5 g. of material, melting-point 212° to 216° C., was obtained.

8. Plant and Animal Poisons possessing a Sterol Skeleton

Plant Heart-poisons

(*a*) Active Principle of Strophanthus

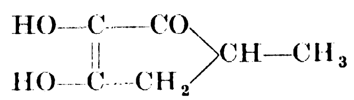
With the aid of chromatography Tschesche and Bohle recently prepared in crystalline form the chloroform-soluble fraction of the active glycoside of commercial strophanthus seed (apparently *S. Preussii* and *S. Barteri*). The substance proved to be identical with the sarmentocymarin, $C_{30}H_{46}O_8$, of Jacobs and Heidelberger.

Five kilograms of the ground seeds were defatted in a Soxhlet apparatus with light petroleum, and then dried in the air and extracted with methyl alcohol. The extract was evaporated on the water-bath and water was added to the residue. The aqueous liquid was extracted with ether, and was then shaken with ten 300-ml. portions of chloroform. The light brown oil (17 g.) remaining after removal of the chloroform was dried in a desiccator and dissolved in 170 ml. of chloroform. An equal volume of benzene was added to the solution and the mixture was poured on to alumina ; the column was developed with a mixture (1 : 1) of chloroform and benzene until the filtrate became colourless. The latter was then evaporated to dryness and the residue was dissolved in methyl alcohol. After being allowed to stand overnight, the solution deposited crystals, which, after one recrystallisation from methyl alcohol and a second recrystallisation from aqueous ethyl alcohol, had melting-point 136° to 137° C.

The saturated lactone prepared by Tschesche and Bohle from sarmentogenin, $C_{23}H_{34}O_5$, a colourless genin obtained from strophanthus, was shown to be identical with the known lactone prepared from digitoxigenin, $C_{23}H_{36}O_2$. The melting-point of the latter was raised to 188°–189° C. by fractional adsorption on alumina.

(*b*) Calotropin, an African Arrow Poison

The toxic principle of *Calotropis procera* (mudar plant) was isolated by Wieland (unpublished observation) and found to possess the approximate composition $C_{29}H_{40-42}O_9$. Hesse and Reicheneder observed that a colourless oil (methyl reductinic acid) distils over when 0·2 g. of calotropin is rapidly heated to 230° C. at 0·05 mm. pressure; this oil crystallised on standing.



Methyl reductinic acid

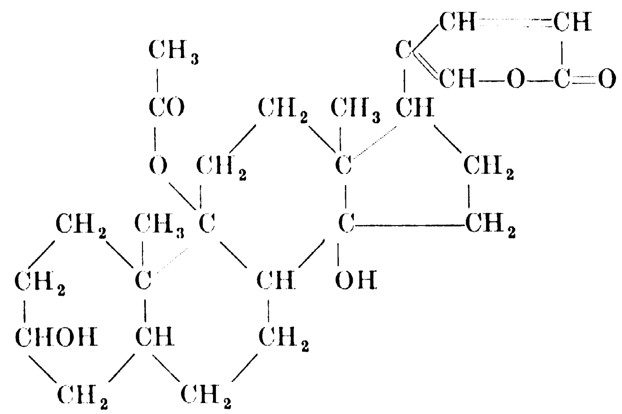
The non-volatile portion, however, resisted all attempts to induce it to crystallise, and so the product from 2 g. of the starting material was dissolved in chloroform and chromatographed on ignited alumina, the column (15 × 1·8 cm.) being developed with chloroform. Two well-separated fluorescent zones were observed when the column was examined in ultra-violet light, and both zones gave a positive Legal's test. Both yielded crystals immediately when the absolute alcoholic eluates were concentrated. The main product, calotropagenin, $C_{23}H_{32}O_6$, obtained from the lower broader zone, formed needles which after several recrystallisations melted at 240° C. The non-fluorescent adsorbent between the two zones did not yield crystals.

Toad Poisons

Bufotalin, $C_{26}H_{36}O_6$, the chief toxin of the common toad (*Bufo vulgaris*), can be isolated more easily and in greater yield by the chromatographic than by any of the older methods (Wieland, Hesse and Hüttel).

The toxin, obtained from 33,000 animals by applying pressure to the parotid glands with forceps and absorbing the secretion (439 g.) on cotton-wool, was dried over phosphorus pentoxide and worked up in three portions. The material was extracted within 4 to 5 days with alcohol-free chloroform in a Soxhlet apparatus and the extract, after being concentrated, was freed from acid by shaking it with *N* sodium hydroxide solution, this treatment usually resulting in the formation of a precipitate. The chloroform solution, together with this precipitate, was poured into a large volume of light petroleum,

and the crumbly precipitate, after drying, was dissolved in acetone and chromatographed on a column of alumina. A



Bufotalin (presumed formula)

single adsorption served to separate quantitatively the bufotalin from the other toad poisons, but it was impossible to effect a fractionation of the latter by further adsorption. An example of the chromatographic separation is as follows.

The dry precipitate formed by pouring the chloroform solution into the light petroleum as described above weighed 35 g.; it was dissolved in 10 volumes of acetone and the solution was filtered and poured on to a column (16 × 7 cm.) of alumina. A yellow pigment present in the solution was found to be adsorbed along with the bufotalin, and so served as an adsorption indicator. The column was developed with chloroform until almost the whole of the yellow pigment washed through, when the column was divided up according to its appearance in daylight and in ultra-violet light, and its behaviour towards Liebermann's reaction. The figures on the left indicate the thickness of the zones (in mm.):

Top:	10	I	Brown zone with blue fluorescence, weak colour reaction (discarded).
	90	II	Colourless zone with green fluorescence. Liebermann's reaction pink to blue to bluish-green.
	40	III	Zone containing the last portion of the yellow pigment. No fluorescence. Liebermann's reaction very similar to that of bufotalin.
Bottom:	20	IV	Liebermann's reaction as with pure bufotalin: red to violet to blue to green.

A mixture of secondary toxins (3·2 g.) was obtained by elution of Zone II with warm chloroform-methyl alcohol

mixture (1 : 1) and subsequent crystallisation of the eluate; melting-point 210° C. Zone III yielded a further 2·2 g. of similar material, whilst Zone IV gave 3·2 g. of bufotalin of melting-point 147° C. The yellow filtrate, however, contained most of the main toxin, and from it 5·4 g. of bufotalin of melting-point (148°) 222° C. and 0·6 g. of melting-point 146° C. were obtained.

From the whole of the secretion, 27·4 g. of bufotalin and 15·3 g. of other toxins were obtained by the above procedure.

Bufotalin can be purified by chromatographic adsorption. Thus bufotalin of melting-point 145° C. was adsorbed on a column from acetone solution and the column was developed with chloroform. A product of melting-point (148°) 222° C. was obtained in a yield of 85 per cent, together with 10 per cent of other toxins.

The mother-liquors, from which nothing more could be induced to crystallise, were concentrated and dried, and the viscous film was dissolved in a little chloroform. The solution was shaken with sodium hydroxide solution and water and then poured into a large volume of light petroleum and the precipitate was dissolved in acetone and chromatographed as above. A further yield of 8·1 g. of bufotalin and 13·5 g. of secondary toxins was thus obtained. These operations can be repeated as many times as is deemed necessary.

The isolation of cinobufagin, $C_{25}H_{32}O_6$, from a Chinese drug "Ch'an Su," which is prepared from the parotid secretions of the Chinese toad (*Bufo gargarizans*), is, according to Tschesche and Offe (1, 2), best accomplished by chromatography.

The material was extracted with chloroform in a Soxhlet apparatus until the solvent was colourless, and the extract was evaporated on the water-bath to a thick oil; benzene was then added so long as a precipitate did not form. The solution was now poured on to a column of alumina, forming 3 to 4 yellow bands in the upper quarter of the column, most of the bufagin being present in the zone around the first band. As the column was developed, these bands slowly travelled downwards and development was continued until a dark zone had reached almost to the lower end of the tube. The golden-yellow filtrate was then evaporated to dryness, and the residue was treated with a little ethyl acetate. On scratching or seeding, the

solution yielded crystals of cinobufagin, which were recrystallised several times from alcohol. A yield of 15 g. of melting-point 223° C. was obtained from 2·5 kg. of the drug. Alternatively, the residue obtained by evaporation of the filtrate could be acetylated.

According to Kotake and Kuwada (1, 2), the cinobufagin, melting-point 220° C., of Ch'an Su is not quite homogeneous. If the chloroform solution is poured on to alumina, the filtrate and the lowest zone of the column yield cinobufagin melting-point 212° to 213° C., whilst from the upper part of the adsorbent "cinobufotalin" can be isolated. The melting-point of the latter is raised from 237°–242° to 248°–249·5° C. by repeating the process.

Wieland's γ-bufogenin can be separated from the alcoholic extract of Ch'an Su by adsorption on alumina from chloroform solution, thus supporting the work of Tschesche and Offe (1, 2).

See also the recent researches of Kondo and Ohno.

For the purification of desacetyl-hexahydro-cinobufagenic acid, see Tschesche and Offe (2).

For data on the adsorption and elution behaviour of snake venom, see Tsuchiya.

9. Heterocyclic Bases. Alkaloids

Tswett's chromatographic method has found frequent application in this field, and even now its possibilities are by no means exhausted. The solution to be chromatographed is prepared either by dissolving a salt of the alkaloid in a solvent mixture containing water, or simply in water, or by dissolving the base itself in an anhydrous solvent. In the latter instance, the use of an acid adsorbent, though not absolutely excluded, is not advisable.

Simple Heterocyclic Substances

The following exploratory experiments were carried out by Kondo.

Separation of Pyridine and α-Picoline

A 1 per cent solution (50 ml.) of both bases in petroleum was chromatographed on a column (20 × 1·5 cm.) of alumina

and the latter was developed with 200 ml. of petroleum. The column was divided up into five parts and each was eluted with a mixture of 20 ml. of ether and 10 ml. of methyl alcohol. The eluates were extracted with dilute hydrochloric acid and the hydrochloride solutions were tested with mercuric chloride solution. The uppermost portion of the column gave a double salt with pyridine, melting-point 176° to 177° C., the third, fourth and fifth portions a double salt with picoline, melting-point 153° to 154° C., whilst both bases were present in the solution from the second portion.

Separation of Quinoline and 8-Hydroxyquinoline

A solution (40 ml.) containing 0·4 g. of the mixture in petroleum was poured on to a column (30 × 1·2 cm.) of alumina, which was developed with ether. Quinoline ran through into the filtrate, where it was characterised as its picrate, melting-point 202° to 203° C. A yellow lake remained on the column and it was impossible to elute this with alcohol.

Separation of Antipyrine and Chloral Hydrate

A solution of 0·7 g. of hypnal (1 mole of antipyrine with 2 moles of chloralhydrate) in 40 ml. of a mixture (4 : 1) of benzene and acetone was filtered through a column (12 × 1·2 cm.) of alumina, which was developed with benzene. Antipyrine, melting-point 113° C., crystallised out from the filtrate.

Separation of Pyramidone and Veronal

The procedure was the same as described in the previous experiment; on developing with benzene, the pyramidone was washed off the column, whilst the veronal was retained in the upper part of the column, from which it was recovered by elution with alcohol.

For the behaviour of carbazole, 1 : 2-benzcarbazole and acridine, see pp. 210–212.

Cinchona Alkaloids

The recovery of cinchonine from 100 ml. of a solution containing 1 part in 1,000,000 of the sulphate was described by Fink (2), who, after adjusting the pH of the aqueous solution

to 8·2 by addition of sodium hydrogen phosphate, filtered it through a mixture (1·5 to 2 g.) of 1 part of asbestos and 3 parts of kaolin. The alkaloid was eluted with dilute sulphuric acid.

Karrer and Nielsen effected the separation of quinine and cinchonine by means of a column of "floridin XXF." A solution of 100 mg. of the bases in a 1 : 1 mixture of chloroform and benzene was chromatographed by using a tube made of uviol-glass, and developing the column with a mixture (4 : 1) of chloroform and benzene. On examination in ultra-violet light, three fluorescent zones were apparent, the upper lilac, the middle with a silvery fluorescence, and the lower brown. The first-mentioned zone gave on elution with boiling alcohol a substance melting-point 208° C., the second another substance melting-point 50° C., whilst the brown zone did not yield any crystalline material. The substance melting-point 208° C. was re-chromatographed, giving three zones, the upper of which gave pure cinchonine, melting-point 254° C. The substance melting-point 50° C. was also re-chromatographed, and likewise gave three zones of which the middle yielded pure quinine, melting-point 56° C. (hydrate), 177° C. (anhydrous).

According to Frehden (unpublished observations), cinchona alkaloids, for instance quinine and cinchonine, can with advantage be separated by chromatographing the chloroform solution on a column of calcium hydroxide. On development with alcohol, a beautiful fluorescent chromatogram is obtained. Other pairs of alkaloids can be separated in a similar manner.

Strychnos Alkaloids

(*a*) Concentration of Strychnine

Fink (2) recovered the strychnine from 100 ml. of a 1 : 1,000,000 aqueous solution by first buffering to *p*H 8·0 with sodium hydrogen phosphate and then filtering through a layer of asbestos-kaolin mixture (1 : 3). The alkaloid remained adsorbed on the filter, from which it was dissolved by dilute nitric acid.

(*b*) The Degradation Products of Vomicidine, $C_{22}H_{26}O_3N_2$

These were purified by filtering the ethereal solution through an alumina column (Wieland and Horner) on which coloured

impurities were adsorbed. A crude oily dehydrogenation product of the base $C_{16}H_{24}O_2N_2$ when thus treated yielded crystals of vomipyrin, $C_{15}H_{16}H_2$, on evaporation of the filtrate. Another dehydrogenation product, $C_{13}H_{17}N$, and a hydrogenation product, $C_{15}H_{20}N_2$, of the base $C_{15}H_{16}N_2$, were similarly purified. The thermal decomposition product of an acid, $C_{19}H_{24}O_7N_2$ (or its ester), obtained by oxidation of vomicidine, was purified by chromatographing the chloroform solution on alumina, but in this instance the crystalline product was not homogeneous.

Opium Alkaloids

The following artificially prepared mixtures were separated on alumina by Kondo.

Morphine and Thebaine

A mixture of the 1 per cent acetone solutions was used and the column was developed with benzene. Morphine was adsorbed towards the top of the column, whilst thebaine, melting-point 191° C., was isolated from the filtrate.

Codeine and Thebaine

Codeine is more easily adsorbed than thebaine. The mixture (0·1 g.) was dissolved in 100 ml. of ether. After developing with 40 ml. of ether, thebaine was detectable in the filtrate.

Narcotine and Thebaine

The material (0·2 g.) was dissolved in a mixture of 60 ml. of benzene and 50 ml. of ether and the solution was poured on to the column (20 × 1 cm.), which was developed with 50 ml. of ether. The filtrate contained 0·1 g. of thebaine and the upper quarter of the column 0·05 g. of crude narcotine, which was eluted with 50 ml. of acetone. The undivided column, when " painted " with strong sulphuric acid, gave an upper green layer due to narcotine and lower down a yellowish-red layer due to thebaine.

Curare Alkaloids

Wieland, Konz and Sonderhoff isolated a new base, toxiferin, from calabash-curare, one of the most potent of the South American arrow-poisons. The crude product, obtained by extraction with methyl alcohol, followed by precipitation with mercuric chloride and decomposition of the precipitate with hydrogen sulphide, was fractionated in the form of its Reineckate (Reinecke's acid is $H(NH_3)_2Cr(SCN)_4$) as follows.

The Reineckate (2·67 g.), obtained by treating 5 g. of the crude material with pure Reinecke's acid, was dissolved in 800 ml. of pure acetone and the solution was run through a 15-cm. column of alumina (100 g.), which was developed with 150 ml. of acetone. Brown inactive impurities remained adsorbed at the top, and the filtrate was concentrated under reduced pressure to a volume of 50 ml. The solution was treated with an equal volume of water and acidified to Congo red by the addition of 2 *N* hydrochloric acid, and the Reinecke acid was removed by extraction with ether. Any Reineckate that tended to separate out during the extraction was re-dissolved by the dropwise addition of acetone and, since the acetone-containing ethereal layer dissolved out some Reineckate, this was concentrated under reduced pressure and the operation was repeated. The reddish-brown aqueous solution remaining after ether extraction was dried over sulphuric acid and sodium hydroxide in a vacuum desiccator, whereupon it formed 0·9 g. of a brown resin, the activity of which was 40 μg per frog unit. This highly active hydrochloride was not crystalline, and was further purified by fractional precipitation with ether from solution in a mixture of alcohol and acetone. It ultimately yielded a well-crystallised anthraquinone-β-sulphonate, having an activity of 20 to 30 μg per frog unit, and a picrate. The constitution is not yet established.

Alternatively, the perchlorate of the crude base can be used for the purification, an acetone solution being poured on to alumina and the column washed with a mixture of acetone and methyl alcohol. The adsorbed perchlorate is retained at the bottom of the column, below a more strongly adsorbed fraction.

Sanguinaria Alkaloids

According to a brief publication by Späth, Schlemmer, Schenck and Gempp, hydroxy-sanguinarin, $C_{20}H_{13}O_5N$, can be isolated from the blood-root (*Sanguinaria canadensis*) by chromatographing the chloroform solution on alumina; melting-point 360° to 361° C. (corr.).

Lupin Alkaloids

Kondo found that *d*-lupanin was more strongly adsorbed on alumina from benzene solution than was sparteine. The column was developed with a 1 : 1 mixture of benzene and petroleum and then with benzene. The lupanin was eluted with methyl alcohol from the top portion of the column and isolated as the picrate, whilst the filtrate yielded crystalline sparteine methiodide.

Sinomenium and Cocculus Alkaloids

Kondo, Tomita and Uyeo isolated from the crude base of *Cissampelos insularis* Makino (Menispermaceæ) a minor alkaloid that appears to be identical with methyl-isochondodendrin, $C_{38}H_{42}O_6N_2$. Two grams of the crude base, freed from phenolic substances by shaking with sodium hydroxide solution, were dissolved in 50 ml. of benzene and the solution was run, drop by drop, on to a 20-cm. column of alumina. The first filtrate contained only traces of a resin, but on developing with benzene an alkaloid fraction began to make its appearance in the filtrate, which crystallised after being concentrated. Development was continued until the filtrate gave no further crystals on concentrating and seeding. The residue left on evaporation of the combined filtrates was dissolved in a little acetone and allowed to stand. The alkaloid crystallised out in the form of long, flat prisms, melting-point 272° to 273° C. (with a red colour). Yield, 0·15 g.

Ergot Alkaloids

The Chemische Fabrik vorm. Sandoz have had considerable success in the separation and purification of the very labile

alkaloids of ergot (*Claviceps purpurea*), but here only the work on ergotamine, $C_{33}H_{35}O_5N_5$, and ergotaminine, $C_{33}H_{35}O_5N_5$, ergotinine, $C_{35}H_{39}O_5N_5$, and its isomer ergotoxine, $C_{35}H_{39}O_5N_5$, will be described.

The sparingly soluble alkaloids ergotaminine and ergotinine, of low physiological activity, are less strongly adsorbed than the readily-soluble alkaloids ergotamine and ergotoxine, which possess a much greater physiological activity. A mixture of ergotamine and ergotaminine, or of ergotoxine and ergotinine, can furthermore be freed from inactive contaminants by adsorption on a column, whilst ergotoxine base has been prepared in crystalline form by the same method. A crude extract can also be resolved into its constituents by chromatography ; finally the substances previously known as "sensibamine" and "ergoclavine" (B.P. 388,529 ; D.R.P. 606,778) have been shown by this technique to be mixtures of known alkaloids.

Isolation of Pure Ergotamine from a Crude Extract

The benzene extract (100 ml.) containing the ergotamine fraction was run through a column of lactose (120 g.) prepared from a suspension in benzene. On washing the column with the same solvent, a filtrate containing an oil but no alkaloid was first obtained, then a filtrate containing traces of dextro-rotatory ergotaminine, and finally the main fraction containing lævo-rotatory ergotamine of a high degree of purity. This portion of the filtrate was evaporated at a low temperature under reduced pressure and the residue was dissolved in aqueous acetone. On standing, the solution deposited colourless rhombic prisms and plates.

Crystalline Ergotoxine from a Crude Alkaloid Mixture

A solution of 1 g. of the alkaloids in 50 ml. of chloroform was run through a column of alumina. After development with chloroform, the initial filtrate was free from alkaloid. On further washing, a dextro-rotatory solution was obtained, from which, according to the source of the raw material and method of working it up, more or less ergotinine was obtained in crystalline form. Later filtrates were lævo-rotary and, on

evaporation at room temperature, gave a residue that crystallised from benzene solution. The crystals were pure ergotoxine base.

Separation of a Mixture of Ergotamine and Ergotaminine

A solution of 0·5 g. of each alkaloid in 100 ml. of chloroform was poured on to a column (40 × 2·2 cm.) of alumina (120 g.), which was then developed with the same solvent. The resulting chromatogram showed zoning when viewed in ultraviolet light. A narrow zone, containing alkaloid, first washed through into the filtrate, and then a zone containing little alkaloid, whilst the upper portion of the column exhibited a brilliant violet fluorescence. The first portion (50 ml.) of the filtrate was dextro-rotatory ($\alpha_D = +8{\cdot}38°$ in a 2 dm. tube) whilst the next 200 ml. of filtrate showed a progressively decreasing optical rotation. After the alkaloid-free fractions there followed a lævo-rotatory filtrate containing ergotamine. The product obtained by evaporation of the dextrorotatory filtrate weighed 0·52 g. ; on being crystallised from pyridine, it gave pure ergotaminine in good yield. The lævorotatory solution gave 0·33 g. of pure ergotamine, recrystallised from aqueous acetone solution.

Separation of a Mixture of Ergotoxine and Ergotinine

A mixture of 0·5 g. of amorphous ergotoxine base and 0·5 g. of crystalline ergotinine was treated as described above. On developing the column, a dextro-rotatory filtrate that yielded well-crystallised ergotinine was first obtained, and this was followed by a lævo-rotatory solution that gave an amorphous residue. This was dissolved in the smallest possible amount of benzene ; on allowing this solution to stand, crystalline ergotoxine base was obtained in good yield, in spite of the fact that the starting material was amorphous.

The Resolution of " Sensibamine " into its Components (1 g. in 100 ml. of chloroform) was effected in a similar way. On developing the column, a dextro-rotatory solution was first obtained, which yielded crystals of ergotaminine from pyridine,

and then a solution containing lævo-rotatory ergotamine, which was crystallised from aqueous acetone. "Ergoclavine" was split up into its components in a similar manner.

Partial Synthesis of Ergobasinine, $C_{19}H_{23}O_2N_3$

Stoll and Hofmann synthesised ergobasine and the isomeric ergobasinine from lysergic acid, $C_{16}H_{16}O_2N_2$. With *d*-2-aminopropanol-1, $HOCH_2.CH(NH_2)CH_3$, this gave an isomorphous mixture of *d*-isolysergic acid-*d*-isopropanolamide and *l*-isolysergic acid-*d*-isopropanolamide which could not be resolved either by crystallisation or by forming salts.

A solution of 0·2 g. of this mixture in 10 ml. of acetone was accordingly poured on to a column (50 × 2 cm.) of alumina moistened with acetone, and the column was developed with acetone. In ultra-violet light all substances containing lysergic acid exhibit a beautiful bluish-violet fluorescence. The initial narrow fluorescent band, however, gradually spread out over the whole column, without separating into zones, so the filtrate was collected in portions, each being evaporated under reduced pressure to a syrup. (A comparatively high temperature was necessary to remove the last traces of solvent, since alumina brings about condensation of acetone—see p. 5.)

The residue from the first portions of the filtrate crystallised from a little acetone on seeding the solution with natural ergobasinine. A yield of 35 mg. of *d*-isolysergic acid-*d*-isopropanolamide was obtained, which after recrystallisation had melting-point 196° C. (corr.) $[\alpha]_D^{20^\circ} = +416^\circ$. The fractions obtained on further washing with acetone were slightly dextrorotatory, but later they became lævo-rotatory, and the final fractions yielded pure, though not crystalline, *l*-isolysergic acid-*d*-isopropanolamide $[\alpha]_D^{20^\circ} = -342^\circ$ Its perchlorate had melting-point 212° C. (corr.).

Amanita Toxin

Chromatography was first used for the investigation of the toxins of the fungus *Amanita phalloides* by Renz, but the following description is taken from a recent publication by

Lynen and Wieland and shows that these toxins appear to occupy a position midway between the simple alkaloids and the complex protein-poisons. In all, three toxins were detected: toxin I, toxin II (phalloidin, crystalline) and toxin III.

1. Separation of the Mixed Toxins

The "primary substance" (27 g.) obtained by pressing the fungi, extracting with methyl alcohol, precipitating with lead acetate and salting out with ammonium sulphate, represented a 30- to 35-fold concentration of the activity. It was dissolved in 80 ml. of water saturated with butyl alcohol and the solution was shaken with four 400-ml. portions of aqueous butyl alcohol. The combined extracts were concentrated under reduced pressure to 110 ml., and immediately treated with just enough water to dissolve the precipitate that had formed during the concentration. One-half the volume of absolute alcohol was then added and the solution was poured on to a column (55 × 3 cm.) of alumina.

(*a*) The column was developed with 250 ml. of a mixture of 68 parts of aqueous butyl alcohol and 36 parts of absolute alcohol; the filtrate yielded 2·850 g. of a substance possessing an activity of 50 μg per mouse unit. It acted rapidly and was free from toxin I.

(*b*) The column was next developed with 150 ml. of absolute alcohol, giving 0·721 g. of a substance having an activity of 25 μg per unit. This was slow acting and was also free from toxin I, but contained the bulk of toxin III.

(*c*) The column was then washed with a mixture (1 : 1) of methyl alcohol and water until the first-formed well-defined zone (greyish-brown above and white below) travelled to the bottom of the column. The filtrate from this washing yielded 6·283 g. of a slow-acting fraction having an activity of 15 μg per unit. This contained toxin I.

(*d*) The column was finally exhaustively extracted with aqueous methyl alcohol, yielding 0·841 g. of a slow-acting fraction with an activity of 90 μg per unit.

In this experiment, toxin I, freed from toxin II and in the main from toxin III also, was obtained in a yield of 70 per cent. By dissolving in water the fractions obtained

from the first two filtrates and seeding, crystals of phalloidin were obtained.

2. Separation of Toxins I and II

A further quantity (33 g.) of the " primary substance " was triturated with absolute alcohol (700 ml.) until no further extraction occurred, the solution being quite colourless. Half of the solution thus obtained, containing 8·4 g. of solid material, was poured on to a column (26 × 3 cm.) of alumina. The column was washed, first with 20 ml., and then separately with 200 ml. of a mixture (9 : 2) of ethyl alcohol and methyl alcohol. The filtrate from the second washing contained the rapidly acting toxin II and had an activity of 50 μg per mouse unit. Toxin I was concentrated in the lower half of the column, though examination in ultra-violet light failed to detect any separation into distinct zones. The toxin was eluted with a mixture (4 : 4 : 1) of methyl alcohol, water and pyridine. The upper half of the column contained only inactive contaminants.

For the estimation of the alkaloid content of galenical preparations, see page 301, and for the estimation of caffeine in coffee, see page 302.

10. VITAMINS

Vitamin A

Detection

This fat-soluble growth factor, which, as is well known, is formed in the animal body from the carotene of the plant food consumed, is abundantly present in the livers of a number of fish, and is the active ingredient of a number of pharmaceutical preparations. The usual method of testing for the vitamin is by means of the Carr-Price reaction, which, however, only gives valid results in the absence of carotenoids. If these are absent or are first removed chromatographically, the position of the vitamin A zone on a chromatogram can be determined by " painting " the column with an antimony trichloride solution in chloroform (Zechmeister, Cholnoky and Ujhelyi, p. 86; Fig. 69, p. 323). This procedure was modified slightly by Willstaedt and With (1); the column was pushed out of the

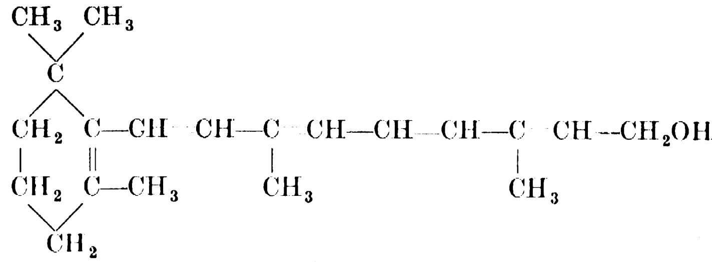
micro-tube and the reagent was dropped on to it from a fine Pasteur pipette. Vitamin A can also be identified, according to Kuhn and Morris, by the " mixed-chromatogram " method (p. 8) using a column of alumina and either petroleum or a mixture of petroleum and benzene as solvent. An ultra-violet lamp is a useful aid.

Isolation

Karrer, Morf and Schöpp extracted the liver oil of halibut (*Hippoglossus hippoglossus*) or mackerel (*Scombresox saurus*) with low-boiling petroleum and saponified the extract by means of 12 per cent alcoholic potassium hydroxide solution at 60° C. in an atmosphere of nitrogen during 1 hour. The unsaponifiable fraction was extracted with light petroleum, and the extract was distilled. The solid residue was freed from sterols by dissolving it in hot methyl alcohol, allowing the solution to stand at − 15° C. for several hours, and then filtering at − 15° C. A further crop of sterols was obtained on allowing the filtrate to stand in the cold room, whilst still further purification was effected by freezing the methyl alcohol solution in solid carbon dioxide and acetone. The vitamin remained in solution, the solid material being discarded. The filtrate was diluted with water and extracted with light petroleum.

These operations effected a considerable concentration of the vitamin and further purification was obtained by chromatography. The light petroleum solution was drawn through a column of fibrous alumina (Merck), when the vitamin was adsorbed in the middle of the column, a small amount of sterols at the top and substances of comparatively low molecular weight at the bottom. The chief zone was eluted with methyl alcohol, the eluted material was transferred to light petroleum and the adsorption was repeated twice. The final extract yielded an unusually pure vitamin A, $C_{20}H_{30}O$, containing 10,500 C.L.O. units. This activity could not be increased by further adsorptions or by acetylation and subsequent regeneration.

Thus chromatography made available an analytically-pure specimen of vitamin A, for which the following formula was established :



Vitamin A

Although no further concentration could be obtained by using alumina, Karrer and Morf (see also Karrer, Walker, Schöpp and Morf) found that, on replacing the alumina by calcium hydroxide, the purification could be pushed a stage further. Two unequal zones were obtained when the product from the previous experiments was put on a column of calcium hydroxide.

Top : β-fraction of vitamin A (main portion). This had a composition agreeing exactly with the formula $C_{20}H_{30}O$, and had an absorption maximum at 325 to 328 mμ ; $E^{1\%}_{1\,cm.}$ (325 mμ) was about 1700. With a solution of antimony trichloride in chloroform it gave a deep blue colour having an absorption maximum at 622 mμ.

Bottom : α-fraction, corresponding only to a few per cent of the material and probably an oxidation product. It contained less carbon, had an absorption maximum at 270 mμ and gave a violet solution with an absorption maximum at 580 mμ when treated with the Carr-Price reagent.

A little earlier than this, Van Eckelen, Emmerie, Julius and Wolff had also obtained a separation of the vitamin into two components by adsorption on fuller's earth.

Heilbron, Heslop, Morton, Webster, Rea and Drummond obtained a very pure preparation of vitamin A by high-vacuum distillation, but found that chromatography scarcely increased the concentration.

In these experiments the apparatus illustrated in Fig. 11, page 60, was used, the air being first displaced by nitrogen. The tube was then filled with a 75-cm. column of alumina, previously ignited at 150° to 200° C. in a current of nitrogen. A solution of 2·5 g. of vitamin A in 25 ml. of light petroleum was slowly run in ; after developing the column with this solvent, it was cut up into three parts, each was eluted with

50 ml. of hot methyl alcohol and the extracted material was transferred to light petroleum after suitably diluting with 200 ml. of water.

For the preparation of vitamin A from the liver-oil of *Theragra chalcogramma*, see Hamano, and for investigations into the vitamin A content of smoked fish, see Willstaedt and Behrnts-Jensen (2).

Karrer's experiments described above resulted in a separation of the very rich concentrate of vitamin A into two components, but similar experiments made by Castle, Gillam, Heilbron and Thompson gave a more complicated result. The solution of 0·5 g. of concentrate in petroleum (boiling-point 60° to 80° C.) was chromatographed on a column (51 × 4 cm.) of calcium hydroxide and this was then washed with several litres of the same solvent. There soon appeared a bright yellowish-brown zone which slowly travelled downwards to about the middle of the column, when it separated into two broad, chocolate-brown zones and several narrow bands (see illustration in the original paper). Altogether five zones were distinguished :

Top : Oxidation products of the vitamin (almost colourless).
Main zone : vitamin A, equivalent to Karrer's β-vitamin A.
A small amount of red material, apparently formed during the actual adsorption and probably consisting of polymers.
A layer corresponding to Karrer's α-vitamin A (" hepaxanthin," probably a decomposition product).
Bottom : Yellow by-product zone, not investigated. The alcoholic solution had absorption maxima at 389, 369, 348 mμ in alcohol.

A very pure vitamin A preparation was obtained by Holmes, Cassidy, Manly and Hartzler using the " liquid chromatogram " method (p. 76). A halibut-liver oil unsaponifiable fraction was dissolved in pentane and the solution was frozen to remove sterols. The filtrate was adsorbed first on norit A (activated in a current of nitrogen) and then on a column of magnesia mixed with one part of the siliceous earth " hyflo super cel " to facilitate filtration.

The apparatus illustrated on page 60, Fig. 12, was used. It was first filled with pure nitrogen and then a thin suspension of norit in pentane was poured into the tube, which was gently

tapped to remove bubbles of gas. In all, 40 g. of adsorbent were used, giving a column 23 × 2·5 cm. A solution of 6 g. of the concentrate in 50 ml. of pentane was poured on to it; the resulting filtrate measured between 50 and 150 ml. By closing the stopcock each time the receiver was changed, and using fresh receivers already filled with nitrogen, the admission of air during the collection of the various fractions was prevented. The final product had a blue value 14,000 times greater than that of the original liver oil.

In a patent granted to Parke, Davis & Co. (assignor Holmes) the preparation of crystalline vitamin A by a similar method is claimed.

Pritchard, Wilkinson, Edisbury and Morton extracted most of the vitamin A from a sterol-free mammalian liver concentrate with 83 per cent alcohol and obtained thereby a vitamin A-active residue containing a substance with an absorption maximum at 285 to 290 mμ. Fractionation of 2·5 g. of this residue on alumina from petroleum solution gave:

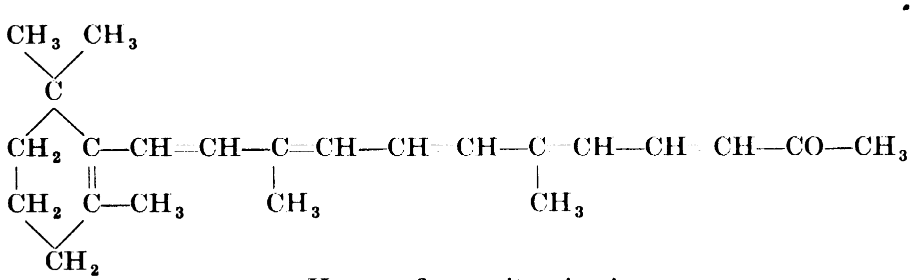
Top:	red	0·726 g.	Iodine value 211	Blue value 2,480
	reddish-yellow	0·489 g.	,, ,, 154	,, ,, 610
	bright yellow	0·5 g.	,, ,, 136	,, ,, 2,630
Bottom:	pale yellow	0·402 g.	,, ,, 129	,, ,, 3,000

No further fractionation could be effected by chromatography and Gillam, Heilbron, Jones and Lederer were similarly unable to purify beyond a certain stage the 693 mμ chromogen ("vitamin A_2").

Ketone from Vitamin A (Batty, Burawoy, Harper, Heilbron and Jones)

A solution of 18·5 g. of a rich concentrate in 500 ml. of pure dry benzene and 200 ml. of acetone (distilled from permanganate and dried with potassium carbonate) was heated under reflux with 20 g. of freshly prepared aluminium tertiary butylate for 30 hours. The deep red suspension was diluted with water and filtered and the benzene layer was washed and dried over sodium sulphate. The solvent was distilled off under reduced pressure, the resulting red oil was dissolved in light petroleum and the solution was poured on to alumina. On washing the column, a reddish-brown zone travelled slowly downwards;

this was eluted with petroleum containing a little methyl alcohol and the extract was washed, dried and distilled. The residue, which still contained vitamin A, was dissolved in a mixture of 200 ml. of absolute alcohol and 20 ml. of glacial acetic acid and the solution was heated under reflux for 1 hour with acethydrazide-pyridinium chloride (Girard's reagent P). The reaction mixture was poured into water and, after 90 per cent of the acid had been neutralised with sodium bicarbonate, the solution was extracted several times with ether. The aqueous phase was treated with a saturated solution of oxalic acid (20 g.) and allowed to stand for half an hour at 50° to 60° C., when the regenerated ketone was extracted with ether. This was an orange-yellow oil that could be distilled in a high vacuum and yielded a crystalline semicarbazone, melting-point 193° C.



Ketone from vitamin A

Separation of Vitamin A and Carotenoids

Karrer and Schöpp showed that vitamin A can be easily separated from either carotene or xanthophyll. As would be expected from its alcoholic nature, vitamin A is more strongly adsorbed than carotene, but less strongly than xanthophyll, which has a longer chromophoric group, and in addition contains two hydroxyl groups.

(*a*) SEPARATION OF VITAMIN A AND CAROTENE : The light petroleum solution is drawn through a column of fibrous alumina and the chromatogram is developed with light petroleum. The upper zone contains only vitamin A ; the lower contains carotene together with a little vitamin A, which can be removed by repeating the adsorption. Elution is accomplished by means of a mixture (9 : 1) of light petroleum and methyl alcohol.

(*b*) SEPARATION OF VITAMIN A AND XANTHOPHYLL (or zeaxanthin and similar carotenoids): The material, dissolved in petroleum or in a mixture of benzene and petroleum, is chromatographed on calcium carbonate. The vitamin A is quantitatively recovered from the filtrate.

Wald used this method with success in separating the vitamin A from the xanthophyll in the pigment obtained from the retina of the bull-frog *Rana catesbiana* (see also Wald and Zussmann). Gillam and Heilbron similarly established the presence of vitamin A in egg-yolk. The ethereal extract was saponified, the sterols were removed, and the residual material was partitioned between light petroleum and 90 to 92 per cent methyl alcohol. The hypophasic pigments were transferred to petroleum by the addition of water, and the solution was chromatographed as above.

Reference should also be made to the paper by Holmes, Lava, Delfs and Cassidy. For the relative behaviour of vitamin A and carotene towards adsorbents, and in particular towards blood-charcoal, see Bowden and Bastow.

The following observation of Emmerie's is of some interest in connection with vitamin A. He found that vigorous treatment with boiling alcoholic potassium hydroxide solution of the fatty-acids obtained from liver oil yielded a yellow amorphous substance, isolated by chromatographing a petroleum solution on alumina, developing the column with alcohol and eluting with dilute alcoholic potassium hydroxide solution. The amorphous product gave a violet colour with the Carr-Price reagent and was obtained in a yield of a few milligrams from 15 g. of fatty acid mixture.

Estimation of the Provitamin A Content of Plant and Animal Material (Zechmeister 5)

The dried, powdered starting material is extracted with ether, and the extract is saponified by allowing it to stand with 30 per cent methyl alcoholic potassium hydroxide solution for 24 hours. The solution is diluted with several volumes of water, and the lower layer is run off. If necessary, the alkali treatment is repeated. The ethereal solution ultimately obtained is washed free from alkali. When the material to be examined is a fat or an organ rich in fat, it can be minced and

treated directly with methyl alcoholic potassium hydroxide solution. The saponification mixture is diluted with water and extracted with ether.

The ethereal extract, after being washed and dried over sodium sulphate, is evaporated and the dry residue is dissolved in light petroleum. The solution is shaken three times with equal volumes of 85 per cent methyl alcohol and the upper layer is then washed free from methyl alcohol with water and, after being dried, is poured on to a column of calcium hydroxide or alumina. On washing the column with petroleum, zones corresponding to the different carotenoids present are formed as follows :

Top :	kryptoxanthin
	lycopene
	γ-carotene
	β-carotene
Bottom :	α-carotene

The column is suitably divided up and each portion is crushed and stirred with petroleum containing a little methyl alcohol. The elution takes place quickly, the suspension is filtered, and the filtrate is freed from methyl alcohol by washing with water. The amount of pigment present in the petroleum solution is estimated colorimetrically by the micro-method of Kuhn and Brockmann (3) or in the Pulfrich photometer.

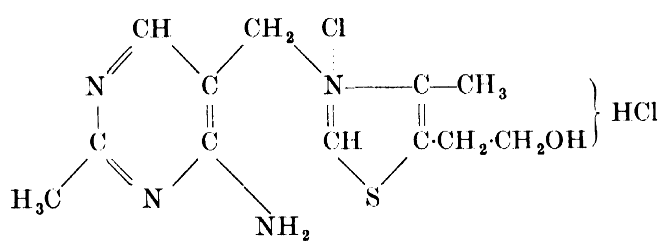
The distribution of the pigments between petroleum and 85 per cent methyl alcohol prior to chromatography is not always necessary, but where this is omitted the chromatogram is more complicated. The separation of the two groups, however, is usually sharp enough for the provitamin to be freed completely from inactive constituents. The anomalous position of kryptoxanthin should be noted.

The proper identification of the various pigment bands is of considerable importance. When the crude material has already been examined in this way and the pigments obtained in crystalline form, then of course identification is easy, as the orientation is known with certainty. If, on the other hand, the material has not previously been investigated, then spectroscopic examination affords valuable, but not necessarily conclusive, evidence of identity. Another useful aid to identification is the "mixed-chromatogram" technique (p. 8).

Considerable difficulties are encountered in attempts at assay in those instances where kryptoxanthin is accompanied by its physiologically inactive isomer rubixanthin, which is fortunately of rare occurrence. The same is true if echinenono is present in a mixture.

Vitamin B_1

The concentration of aneurin from extracts of rice-polishings, brewer's yeast or wheat germ was successfully accomplished by Cerecedo and Hennessy, by Cerecedo and Kaszuba, and by Cerecedo and Thornton, using filtration through artificial zeolite. Thus, for example, the commercial preparation " Decalso " (The Permutit Co., New York), after being stirred several times in water and then in sulphuric acid of pH 4·0, was packed to a height of 46 cm. in a metal tower (150 × 28 cm.)



Vitamin B_1

and heated to a temperature of 75° C. by passing hot water through it. The extract (300 litres; pH 4·5) from 30 kg. of rice-polishings, obtained by a preliminary purification treatment with baryta, was heated to 75° C. and run through the column. It passed through at the rate of 1 litre per minute, and was followed by four 38-litre portions of hot water. In this way all the aneurin from the extract was retained by the zeolite, and was eluted by pouring 76 litres of a hot molar solution of ammonium nitrate through the column. This ran through at the rate of ½ litre per minute, and when elution was complete, the column was washed with 10 litres of hot water. The filtrate, after cooling, was adjusted to pH 7·5 with ammonia and the aneurin was isolated by way of its silver salt. A yield of 40 mg. of pure aneurin was obtained.

Detection and Estimation

Willstaedt (11) and Willstaedt and Bárány found that vitamin B_1 coupled with diazotised 2 : 4-dichloroaniline to give a yellowish-red, ether-soluble dyestuff, which was adsorbed from ether solution on a column of calcium hydroxide. The apparently uniform, reddish-violet pigment was eluted with alcohol. The reagent was prepared by heating 0·8 g. of powdered 2 : 4-dichloroaniline with 10 ml. of conc. hydrochloric acid, dissolving the product in hot water and diluting to 500 ml. ; 10 ml. of this solution were mixed immediately before use with 20 ml. of a 0·1 per cent sodium nitrite solution. Five millilitres of the diazonium solution so obtained, and 1 to 2 ml. of 20 per cent sodium hydroxide solution were added to 20 to 70 ml. of the vitamin B_1 solution containing 40 to 100 μg of the vitamin. After standing for 20 minutes, 1 ml. of conc. hydrochloric acid was added, the solution was extracted with ether, and the ethereal extract was dried over sodium sulphate.

The formation of the dyestuff was utilised for the detection of vitamin B_1 by the " brush " method. Thus aneurin was adsorbed on a column of alumina from aqueous solution and the column was painted with the diazonium solution made alkaline with sodium carbonate.

Widenbauer, Huhn and Becker, in certain experiments on the detection of vitamin B_1 in urine, found that the conversion of the vitamin into the blue-fluorescent substance thiochrome by oxidation with alkaline potassium ferricyanide solution was apparently prevented by the pigments of urine. If, however, the urine at pH 5 was filtered through a column of alumina (Merck), washed before and after the filtration with water at the same pH, the pigments were adsorbed at the top of the column, which gave no reaction when painted as described above. The middle zones gave a strong positive reaction, whilst the lowest part of the column gave no reaction. When the inhibiting power of these various zones was tested by means of an artificially prepared solution of vitamin B_1, it was found that the coloured zones at the top had a very marked inhibitory effect. The vitamin can frequently be estimated fluorometrically by first removing these interfering pigments

chromatographically, but the method cannot be said to be of clinical value as yet.

Vitamin B_2 (Lactoflavin)

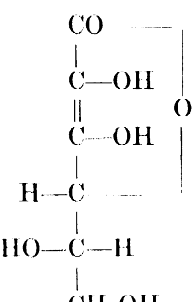
This has already been discussed, along with the other pigments (p. 163 *et seq.*), where its structural formula is given.

Vitamin C

The antiscorbutic vitamin, *l*-ascorbic acid, as is well known, was isolated by Szent-Györgyi.

Isolation of the 2 : 4-Dinitrophenylhydrazone of Dehydro-ascorbic acid from Urine

The following directions are given by Drumm, Scarborough and Stewart. Twelve litres of fresh urine were treated with sufficient oxalic acid to give a concentration of 4 per cent of the acid, and the liquid was then concentrated to 2 litres in an atmosphere of carbon dioxide and filtered on the following day.



Vitamin C

The filtrate was treated in the cold with two 25-g. portions of norit, which, besides clearing the solution, oxidised the vitamin C to dehydro-ascorbic acid ; the preparation of the hydrazone could have been carried out on the resulting solution, had it not been desired to obtain analytical data at the same time. The straw-yellow solution was therefore allowed to stand for 48 hours in an atmosphere of hydrogen sulphide, in order to reduce the dehydro-ascorbic acid back to ascorbic acid. The

hydrogen sulphide was removed with carbon dioxide and the excess of oxalic acid was precipitated by the addition of a thin paste of calcium hydroxide (60 g.). The solution was finally made neutral with solid calcium carbonate, and after a further treatment with hydrogen sulphide, hydrochloric acid was added to make it 2·5 N ; it was then titrated with N iodine solution until a pale blue colour was produced with starch solution.

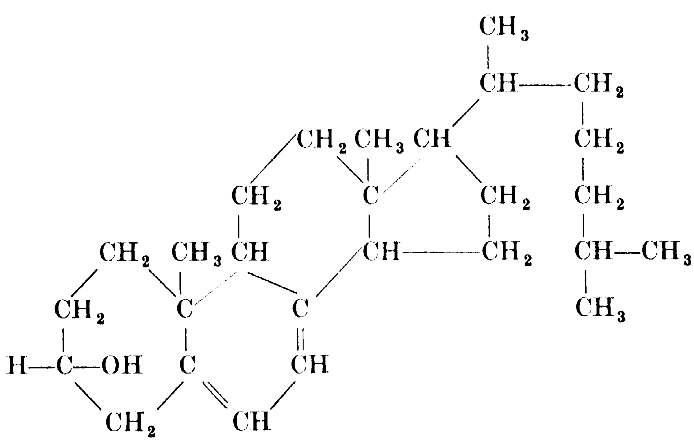
To the solution of dehydro-ascorbic acid thus obtained a solution of 16 g. of 2 : 4-dinitrophenylhydrazine in 500 ml. of hot 2·5 N hydrochloric acid was added ; the mixture was allowed to stand for 4 days at 40° C., and then to cool during 24 hours. The reddish-brown precipitate was filtered off, digested twice with hot 2·5 N hydrochloric acid and then washed with water until free from acid, and dried in a vacuum desiccator. The hydrazone (4 g.) was extracted with a 1 : 1 mixture of absolute alcohol and absolute acetone, and the solution was poured on to 5 columns (14 × 3 cm.) of alumina, previously saturated with the same solvent mixture. A narrow brick-red band was formed at the top of each column, and below this a broad, deep-purple region, the two zones being fairly well differentiated. The columns were washed with acetone until the filtrate was colourless, and the whole of the coloured alumina from the 5 columns was combined and eluted with hot glacial acetic acid ; the eluates were concentrated under reduced pressure. The reddish-purple precipitate that formed on standing overnight was filtered off and washed with water. The yield was 1·4 g. By concentrating the mother-liquors, or pouring into water, a further 1·62 g. of the product was obtained. The acetone-alcohol extract from the combined precipitates was chromatographed a second time and gave a chromatogram similar to that described above. Each coloured zone was separately eluted and the acetic acid solutions were precipitated with 2 volumes of water and allowed to stand for 3 days. The precipitates were re-chromatographed separately, and the purple zones from each of the columns were combined and eluted. The material recovered from the eluate as before was dissolved in acetone-alcohol mixture and adsorbed on a fourth column, and the broad purple zone was extracted with glacial acetic acid. The extract yielded 20 mg. of pure hydrazone, melting-point 279° C. (decomp.) after recrystallisation

from a mixture of acetone and alcohol. It had the composition represented by the formula $C_6H_6O_4[N.NH.C_6H_3(NO_2)_2]_2$.

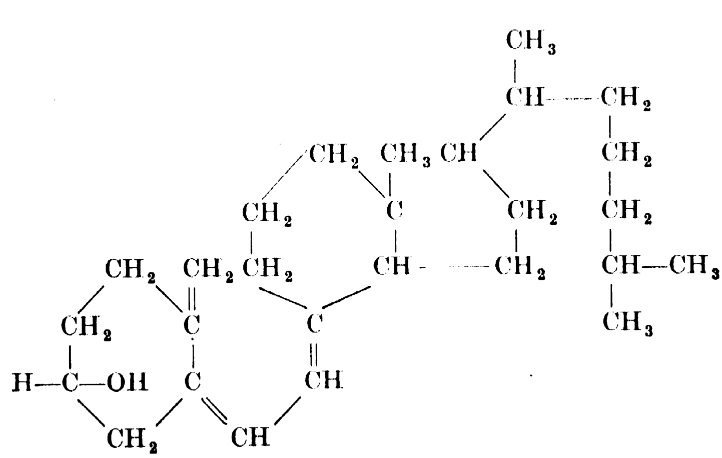
The isolation by chromatography of another dinitrophenylhydrazone, not yet identified, is described in the original publication.

Vitamin D

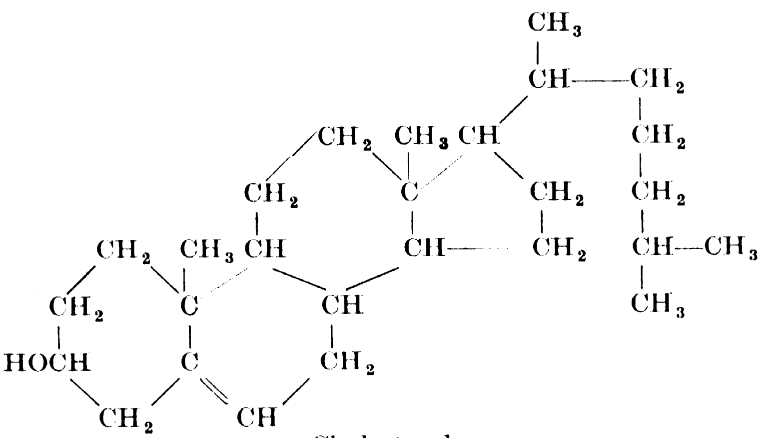
The antirachitically active vitamin D_3 was isolated from the irradiation product of 7-dehydrocholesterol by Windaus, Schenck and Werder, simultaneously with its isolation from tunny-fish and halibut liver oils by Brockmann (3, 4). Chromatography was employed in both instances. (See also p. 257 and the uncompleted work of Neracher and Reichstein.)



7-Dehydro-cholesterol

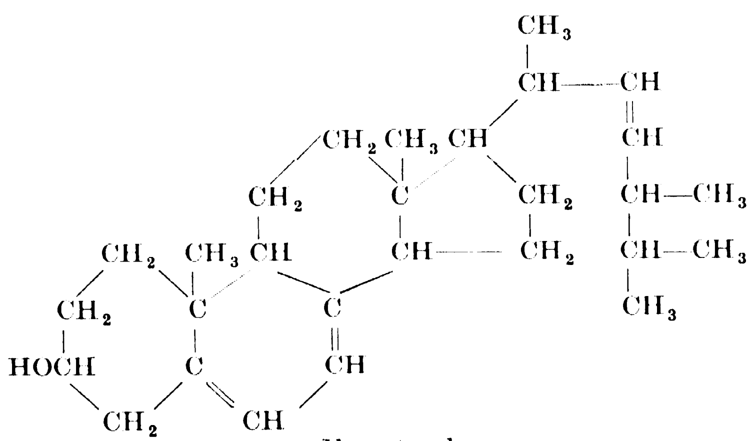


Vitamin D_3



Cholesterol

(One double bond, weak adsorption affinity)



Ergosterol

(Three double bonds, stronger adsorption affinity)

The investigations on fish liver oils are of considerable interest from the practical point of view. For instance, the vitamin could not be induced to crystallise and it was therefore converted into an ester. Even this crystallised only after being chromatographed. The vitamin was obtained in a state of purity by hydrolysis of this crystalline ester. The preliminary stages in the isolation of the vitamin from tunny-fish liver oil consisted of repeated fractionations, followed by a series of very effective chromatographic adsorptions. Another interesting feature of this work is the use of a dyestuff to indicate the whereabouts of the vitamin D_3 zone. The method used by Brockmann (3) also resulted in a separation of the vitamins A and D of the liver oil at the same time.

(*a*) **Vitamin D_3 from 7-Dehydrocholesterol** (Windaus, Schenck and Werder)

A benzene solution of 6·3 g. of 7-dehydrocholesterol was irradiated for 3¾ hours in a small quartz tube with light from a magnesium spark. After distilling off the benzene under reduced pressure, the residue was dissolved in 60 ml. of alcohol; the solution was treated with a solution of 8 g. of digitonin in 120 ml. of boiling alcohol and evaporated to dryness without removal of the precipitate. The residue was extracted several times with boiling light petroleum and the extract was evaporated and treated with 15 ml. of light petroleum and 2 ml. of citraconic anhydride, followed by sufficient absolute ether to give a clear solution. After standing for 6 days at 20° C., 40 ml. of 10 per cent methyl alcoholic potassium hydroxide solution were added, and the mixture was allowed to stand for a further 12 hours. It was then diluted with water and extracted with light petroleum. The solvent was evaporated off under reduced pressure, leaving behind a bright yellow resin which showed no tendency to crystallise. The 3 : 5-dinitrobenzoate also proved refractory.

The resin was dissolved in a 4 : 1 mixture of petroleum and benzene and chromatographed on alumina. The column was cut into three parts and each was eluted with benzene containing a little methyl alcohol. Each eluate was esterified with 3 : 5-dinitrobenzoyl chloride and pyridine. Yellow needles of vitamin D_3-dinitrobenzoate, $C_{34}H_{46}O_6N_2$, gradually crystallised out from the fraction recovered from the middle zone of the column. After several recrystallisations, the substance had melting-point 129° C. Hydrolysis of these crystals with methyl alcoholic potassium hydroxide solution gave the free vitamin in the form of an oil, which had the same absorption spectrum as vitamin D_2, and had antirachitic activity at a dose of 0·05 μg when tested on rats.

(*b*) **Vitamin D_3 from Tunny-fish and Halibut Liver Oils** (Brockmann 3, 4)

All the solvents used were carefully purified and after each fractionation the solvent was removed in a desiccator evacuated to 1 mm. four times, oxygen-free carbon dioxide being admitted after each evacuation.

In the description given below, " A " denotes the vitamin A content expressed in cod liver oil (CLO) units, and " D " denotes the vitamin D content in international units per milligram.

Batches of tunny-fish concentrate (vitamin D content 0·32 per cent ; A = 2,900 ; D = 80) each weighing 100 g. were dissolved in 2 litres of petroleum and the solution was shaken sixteen times with 500-ml. portions of 90 per cent methyl alcohol. The petroleum layer contained 50·5 g. of material, A = 580 ; D = 140. It was diluted to 1·5 litres with petroleum and shaken twelve times with 500-ml. portions of 95 per cent methyl alcohol. The combined alcoholic solutions were diluted with water so that the alcohol concentration was 50 per cent, and the solution was then extracted with petroleum, giving an extract that contained 30 g. of substance, A = 500, D = 280.

Twenty grams of this last preparation were dissolved in a mixture of 100 ml. of benzene and 400 ml. of petroleum, the solution was filtered through a column of " aluminium hydroxide III," and the column was washed with 1·2 litres of solvent. The filtrate contained 5·8 g. of oil, A = 550, D = 800.

Ten grams of the material from the filtrate were dissolved, together with 100 mg. of Indicator red 33, in 600 ml. of a 1 : 4 mixture of benzene and petroleum, and the solution was filtered through a second column of " aluminium hydroxide III." After this had been washed with 3 litres of solvent, a chromatogram was formed having the following appearance :

Top :	bright yellow
	pink
	red, containing 2·9 g. of oil (D = 2,000) and Indicator red
Bottom :	brownish-yellow

This oil (2·9 g.) obtained from the column was dissolved in 200 ml. of a 1 : 4 mixture of benzene and petroleum and filtered from a little Indicator red that had crystallised out, and the chromatography was repeated as before. After washing with 1·5 litres of solvent, the following chromatogram was obtained :

Top :	bright yellow
	red, containing 0·9 g. of oil (D = 5,500) and Indicator red
Bottom :	yellow

The oil (0·9 g.) was freed from traces of solvent and taken up in 50 ml. of petroleum. The solution was then shaken with 20 per cent aqueous methyl alcoholic (80 per cent) potassium hydroxide solution until the petroleum layer was free from colour. The oil obtained after removal of the petroleum was dissolved in a little hot methyl alcohol ; on cooling, the bulk of the cholesterol present crystallised out. The filtrate was warmed with digitonin and evaporated to dryness, after which the residue was extracted with hot petroleum. Evaporation of the petroleum yielded a cholesterol-free fraction, D = 6,700.

The latter product (750 mg.) was dissolved in 4 ml. of pure pyridine (dried over barium oxide) and treated with a solution of freshly distilled 3 : 5-dinitrobenzoyl chloride in 3 ml. of pyridine. After warming for a short time, the mixture was allowed to stand for 2 days at 20° C. in an atmosphere of carbon dioxide and was then poured into sodium bicarbonate solution. The aqueous liquid was extracted with benzene and the extract was washed successively with sodium bicarbonate solution, dilute acetic acid and water. It was then evaporated and the residue was dissolved in 70 ml. of a 1 : 4 mixture of benzene and petroleum ; the solution (from which a small amount of insoluble material separated) was poured on to a column of "aluminium hydroxide III." This was washed with 70 ml. of solvent, 4 zones being formed. The eluate from the lowest of these after removal of the solvent gave an oil, D = 10,000. The oil was dissolved in a little hot acetone, and methyl alcohol was added until a slight turbidity was produced. The oily precipitate that first separated out crystallised on standing, forming clusters of yellow needles. After some time, the mother-liquors were poured off and the solid was freed from oil by washing with low-boiling petroleum. The crystals, after being twice recrystallised from a mixture of acetone and methyl alcohol, had melting-point 128° to 129° C., identical with that of the ester described in (*a*). Hydrolysis was effected by warming for half an hour with 5 per cent methyl alcoholic potassium hydroxide solution in an atmosphere of nitrogen, and the product was oily vitamin D_3 with a biological activity of 25,000 international units per milligram.

For halibut liver oil the same procedure was adopted as with tunny-fish liver oil, a concentrate from 15 kg. of oil,

D = 120, being used. The 3 : 5-dinitrobenzoate that resulted was identical with the foregoing.

(c) Isolation of Vitamin D_3 from Blue-fin Tuna Liver Oil (Brockmann and Busse)

In general, the method as described above was employed, except that one of the partition processes between petroleum and 95 per cent methyl alcohol was omitted. Instead, the residue left on evaporation of the petroleum solution was repeatedly extracted with cold 99 per cent methyl alcohol, which removed the vitamin, whilst leaving the hydrocarbons behind in the oil. The methyl alcohol solution was evaporated and the residue was used for the adsorption.

The unsaponifiable fraction (285 g.) from 5 kg. of liver oil, D = 100, was concentrated as described above. Thus 98 g. were dissolved in 2 litres of petroleum and the solution was extracted 16 times with 500-ml. portions of 90 per cent methyl alcohol. The petroleum solution contained 50·3 g. of substance, D = 200, of which 28·2 g., together with 0·5 g. of Indicator red 33, were dissolved in 350 ml. of a 1 : 4 mixture of benzene and petroleum and chromatographed on "aluminium hydroxide III." After developing with 800 ml. of solvent, the following chromatogram was obtained :

Top :	reddish-brown
	red, containing 6·3 g. of oil (D = 800)
	brown
Bottom :	bright yellow

The combined eluates (20·6 g.) from the red zone of several chromatograms were re-chromatographed from the same solvent. The second chromatogram after washing with 6 litres of solvent was as follows :

Top :	bright yellow
	red, containing 9·65 g. of oil (D = 1,600)
Bottom :	brownish-yellow

The oil was chromatographed a third time (together with 0·2 g. of Indicator red) from the same solvent (600 ml.) and washed with 3·5 litres of solvent.

Top :	yellowish-red
	red, containing 3·11 g. of oil after removing the Indicator red (D = 4,000)
Bottom :	brownish-yellow, containing 0·863 g. of oil (D = 4,000)

The sterols and the remaining Indicator red were removed from the combined active fractions, and the product was esterified with 3 : 5-dinitrobenzoyl chloride. The crude ester was chromatographed on "aluminium hydroxide III" from a mixture of acetone and methyl alcohol; the lowest zone yielded an oil that crystallised from a mixture of acetone and methyl alcohol. After one recrystallisation from acetone the ester had melting-point 128° to 129° C.

Isolation of the Provitamins D

The provitamins ergosterol and 7-dehydrocholesterol (see the structural formulæ given on pp. 255–256) contain respectively 3 and 2 double-bonds; they are both more strongly adsorbed than cholesterol and similar sterols having one double-bond.

(*a*) The ergosterol[1] in commercial cholesterol was concentrated by Winterstein and Stein (1) by chromatographing 20 g. of cholesterol (containing 5·6 mg. of ergosterol) dissolved in 300 ml. of a 1 : 1 mixture of petroleum and hexane on a column (15 × 5·5 cm.) of activated alumina. After developing with the same solvent mixture it was cut up into 5 equal parts, and the amount of ergosterol in each was determined spectrophotometrically:

Top:	Zone I, yielded 4·1 g. containing 4 mg. of ergosterol
	Zone II, yielded 4·55 g. containing 1·6 mg. of ergosterol
Bottom:	Zones III–V, yielded about 10 g., free from ergosterol

Thus in Zone I the ergosterol was concentrated 3·5-fold by one adsorption. The degree of concentration was increased to 6-fold by a second adsorption.

(*b*) ARTIFICIAL MIXTURE: Karrer and Nielsen poured a benzene solution containing 50 mg. of cholesterol and 100 mg. of ergosterol on to a column of alumina and developed with 1 litre of benzene. In ultra-violet light, several fluorescent zones were observed, which were separately eluted with boiling ethyl alcohol-benzene mixture (3 : 7). The residues remaining after evaporation were crystallised from ethyl alcohol. The following results were obtained:

[1] Note. This substance is more likely to have been 7-dehydrocholesterol than ergosterol.—TRANSLATORS.

Top :	I	yielded no material
	II	yielded a small amount of substance, melting-point 149° C.
	III	yielded a substance melting-point 145° C.
	IV	yielded a substance melting-point 139° C.
	V	yielded only a trace of material
Bottom :	VI	yielded nothing

The sterols isolated from zones III and IV were re-chromatographed, and the uppermost zone from this second column gave a product of melting-point 156° C. (ergosterol melts at 157° C.). The second zone gave a substance melting-point 143° C. (cholesterol melts at 146° C.). The ergosterol was quite pure, but the cholesterol fraction contained a small amount of ergosterol.

(*c*) According to a patent of Philips Gloeilampenfabriken, the provitamin can be isolated from a naturally occurring sterol mixture by chromatographing the acetate on alumina from a 1 : 1 mixture of benzene and light petroleum. The column is eluted with a mixture of benzene and methyl alcohol and the eluate is recrystallised from methyl alcohol. The ergosteryl acetate so obtained had melting-point 160° to 161° C., and the free sterol melting-point 149·5° to 150° C.

(*d*) ERGOSTEROL FROM THE STEROLS OF EGG-YOLK (Windaus and Stange) : The cholesterol (5 kg.) from Chinese dried egg-yolk was used as the starting material. From the absorption band it was calculated that the provitamin content of the sterol was 0·18 per cent. Samples, each weighing 35 g., were dissolved in 460 ml. of a 1 : 1 mixture of petroleum and benzene and the solution was poured on to a column (65 × 5 cm.) of alumina (moisture content 3 per cent). The column was washed with a mixture of petroleum, benzene and methyl alcohol (250 : 250 : 1·2) until no more cholesterol appeared in the filtrate, a total of 14 to 15 litres of solvent being required for this purpose. The first 10 litres of filtrate, collected during 22 hours, contained 25 g. of cholesterol ; the next 1·5 litres contained 6 g. having a provitamin content of 0·5 per cent, whilst the last 2 litres contained 4 g. having a provitamin content of 1·2 per cent. After a few months' work, 250 g. of this last fraction had been accumulated and, on re-treatment, yielded 35 g. of cholesterol with a provitamin content of 4·5 per cent. Chromatography of this material yielded 4·2 g.

of sterol, containing 19·4 per cent of provitamin, and this in turn yielded 0·745 g., containing 81·4 per cent, and finally 0·423 g. of pure ergosterol. Recrystallised once from a mixture of ether and methyl alcohol, it had melting-point 163° C.

(*e*) ERGOSTEROL FROM COTTONSEED OIL (Windaus and Bock 2): The crude brown sterol (45 g.) was treated as described above, but in this instance the provitamin was separated in a single operation from other sterols and impurities. The first 11 litres of filtrate were discarded, but the twelfth litre yielded 0·93 g. of 95·5 per cent pure provitamin, whilst subsequent fractions contained hardly any. The purified provitamin was identical with ergosterol

(*f*) 7-DEHYDROCHOLESTEROL FROM PIG'S SKIN (Windaus and Bock 1): From 100 kg. of the skin, 30 g. of crude sterols were obtained, containing 4 per cent of provitamin. These were adsorbed on alumina from a 1 : 1 mixture of petroleum and benzene, and the column was washed with a mixture (250 : 250 : 1·2) of petroleum, benzene and methyl alcohol, the filtrate being collected as usual in fractions (see accompanying table).

Litres of Filtrate	Sterol Content (%)	Provitamin Content (%)
10	4·3	0
3	10·92	0·1
1	6·60	0·5
0·5	3·23	4·3
0·5	2·42	8·7
0·5	1·73	17·3
0·5	0·73	71·0

After two further chromatographings on small columns, the last fraction yielded 0·379 g. of sterol with a provitamin content of 91 per cent. Purified by way of its 3 : 5-dinitrobenzoate, it gave pure 7-dehydrocholesterol. The acetate had melting-point 130° C.

(*g*) LUMISTEROL$_3$, an irradiation product of 7-dehydrocholesterol, was purified in the form of its dinitrobenzoate by filtering a benzene solution through alumina. The filtrate, which was colourless, yielded pure lumisteryl$_3$-dinitrobenzoate. Lumisteryl$_3$-acetate was purified in a similar manner (Windaus, Deppe and Wunderlich).

Vitamin E

According to Drummond, Singer and MacWalter (1, 2), the antisterility vitamin can be prepared in the form of a concentrate by fractional adsorption of the unsaponifiable matter of wheat-germ oil. Thus 33 g. of the yellow oil, from which most of the sterols had been removed, were dissolved in 500 ml. of light petroleum. On standing, lutein crystallised out and the filtrate was chromatographed on alumina. On washing the column with large volumes of light petroleum, a colourless filtrate was first obtained, whilst a yellow zone, containing kryptoxanthin, travelled slowly downwards and eventually passed out of the column into the filtrate. Both these fractions were inactive. At this stage the column exhibited the following zones :

Top :	brownish-yellow, biologically inactive
	sandy-yellow, almost inactive at a dose of 2 to 5 mg.
	dark coloured, feebly active
Bottom :	pale coloured, the most active fraction, possessing activity in a dose of 1 mg. per day.

By repeating the adsorption of the two lowest zones several times, the concentration of the antisterility factor was further increased, and material active in a dose of 0·1 mg. per day was obtained. Cottonseed oil behaved in a similar manner. In neither instance was the product homogeneous.

For another account of the chromatography of vitamin E, see the publication of Karrer and Salomon (2), which confirms the work of Drummond, Singer and MacWalter. See also under "sterols," page 221.

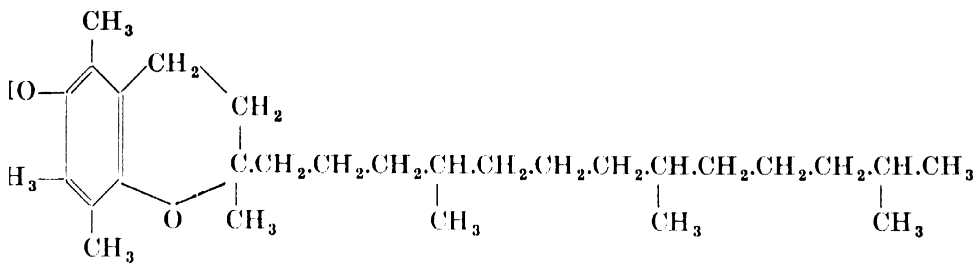
According to the latest researches, wheat-germ oil, as well as other similar oils, contains several vitamin E-active substances, the most active of which is called α-tocopherol, $C_{29}H_{50}O_2$. Evans, Emerson and Emerson isolated this substance in the form of its allophanate. The free alcohol prevented sterility in rats in a single dose of 3 mg. The authors tested the homogeneity of their allophanate (melting-point 156° to 157° C.) chromatographically.

A 3·5-cm. tube was filled with 100 g. of calcium carbonate (previously heated to 150° C. for 3 hours), a solution of 106 mg. of the substance in a little benzene was poured on to it and the column was washed with 1,150 ml. of benzene. The column,

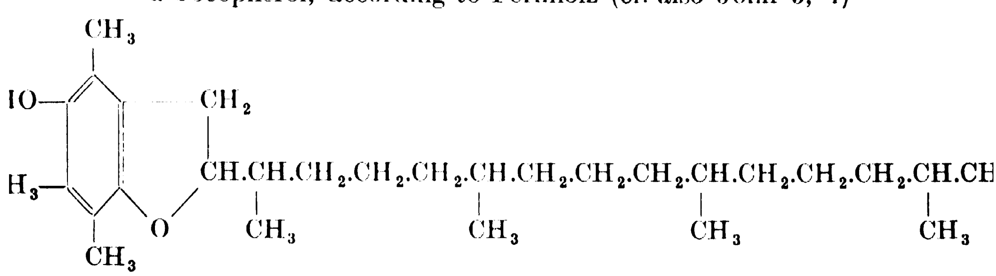
cut into three parts, yielded 26 mg. of allophanate, and the most strongly adsorbed fraction was just as active in the rat-test as the material recovered from the filtrate.

A second active substance, called β-tocopherol, was isolated as the allophanate by a similar procedure. The method had already been the subject of a patent taken out by the I.G. Farbenindustrie.

According to John (2), β-tocopherol is the next lowest



α-Tocopherol, according to Fernholz (cf. also John 3, 4) [1]



α-Tocopherol, according to Karrer, Salomon und Fritzsche

homologue ($C_{28}H_{48}O_2$) of the α-compound, and is identical with his so-called cumo-tocopherol, and also apparently with the neo-tocopherol of Karrer, Salomon and Fritzsche. Mention should also be made of the γ-tocopherol of Emerson, Emerson, Mohammad and Evans (see also Drummond and Hoover). A recent summary is given by John (4).

Examples of Chromatography applied to the Tocopherols

(*a*) Separation of the Allophanates of two Vitamin E-Active Alcohols (I.G. Farbenindustrie) : Ten grams of a vitamin E preparation, obtained by the removal of most of the sitosterol from the unsaponifiable matter of wheat-germ

[1] This chroman formulation is now universally accepted. β-Tocopherol appears to be the 1 : 4 dimethyl and γ-tocopherol the 3 : 4 dimethyl compound.—TRANSLATORS.

oil, are dissolved in 250 ml. of dry light petroleum. Cyanic acid prepared from 30 g. of cyanuric acid is led into the solution, which is strongly cooled. After standing overnight, it is evaporated to dryness and extracted with ether. The residue from the ethereal extract is taken up in 100 ml. of light petroleum, whereupon a little sitosterol allophanate remains undissolved. The solution is now drawn through a column (15 × 2 cm.) of alumina and the latter is washed with 500 ml. of light petroleum. The column is sucked dry and eluted with a mixture (4 : 1) of methyl alcohol and ether. On concentrating the solution, there crystallises first a sparingly soluble allophanate of melting-point 160° C., and on further concentration of the mother-liquors, a readily soluble isomeric allophanate of melting-point 135° to 138° C. The alcohol prepared from the allophanate of melting-point 160° C. by saponification has the composition $C_{29}H_{50}O_2$ and contains 1 rat unit in 2 to 3 mg.

In the above patent specification it is emphasised that the chromatographic method can be applied, not only to the allophanate, but also to other crystallisable derivatives prepared from the alcohols by means of acid chlorides, acid anhydrides, etc.

(*b*) **Isolation of α-Tocopherol** (Karrer and Salomon 2): The " Fraction B " obtained from wheat-germ oil according to the procedure described on page 222 contained almost all the α-tocopherol originally present. After removal of the light petroleum by distillation, an orange-coloured oil remained, 30 g. of which were adsorbed in the usual manner on alumina. A second chromatographic treatment was carried out with the object of removing feebly adsorbed vitamin E-active constituents. The petroleum washings from this second chromatogram, however, came through yellow almost immediately, and the washing was therefore discontinued as soon as the filtrate became colourless, in order to avoid loss of tocopherol, appreciable quantities of which were otherwise washed off the column. About 3 litres of light petroleum were usually sufficient for 30 g. of oil, most of the inactive substances being thereby removed, whilst the filtrate contained but little tocopherol, as shown by its low reducing-power when treated with methyl alcoholic silver nitrate solution.

The chromatogram, prepared from a column 70 cm. long, was not well differentiated, and consisted of a pale yellow adsorbate that was darker in colour towards the bottom, and a discoloured zone 2 to 3 cm. wide at the top. The latter was rejected. Since the α-tocopherol was distributed quite evenly over the rest of the column, this was eluted, without being divided up, with a mixture (4 : 1) of methyl alcohol and ether. The eluate was distilled under reduced pressure, the residue was dissolved in light petroleum, and the solution was shaken several times with water, dried over sodium sulphate and distilled under reduced pressure with careful exclusion of moisture. Each 10-g. portion of the oil was dissolved in 250 ml. of dry benzene and the solution was saturated with cyanic acid prepared from 15 g. of cyanuric acid. After standing at 5° C. for 10 to 12 days, the reaction product was run through a sintered glass filter and the precipitate was washed with warm benzene. The filtrate was evaporated under reduced pressure and the resulting oil was dissolved in hot acetone (60 to 70 ml. for each 10 g.) and filtered while still hot to remove a little jelly. Crystals began to separate from the filtrate in a very short time, and after 24 hours the yellowish solid was filtered off, giving a yield of 1·5 to 1·75 g. from 10 g. of the oil from "Fraction B." The crude material had melting-point 152° to 154° C. Recrystallised twice from ethyl acetate, the α-tocopheryl allophanate had melting-point 159° to 160° C.

Fernholz chromatographed the thermal decomposition products of α-tocopherol, obtained by heating 2·1 g. at 335° C. for 6 hours. In the neck of the retort, 0·257 g. of durohydroquinone collected, together with a red liquid. The latter was diluted with 100 ml. of light petroleum and filtered through a 20-cm. column of activated alumina, which was afterwards washed with the same solvent. The now colourless filtrate yielded 1·17 g. of a hydrocarbon $C_{18}H_{36}$ on evaporation.

(*c*) **Isolation of β-Tocopherol** (Todd, Bergel and Work): A concentrate (250 g.) of the unsaponifiable fraction of wheat-germ oil, having activity in a dose of 125 mg., was dissolved in petroleum (boiling-point 100° to 120° C.) and washed with 92 per cent methyl alcohol. It was then extracted 10 times with 500-ml. portions of absolute methyl alcohol according to the method of Evans, Emerson and Emerson. The oil

obtained from the methyl alcohol extract was dissolved in 750 ml. of methyl alcohol and allowed to stand for 24 hours at 0° C. The dark semi-crystalline mass (40 g.) that separated was filtered off, and the filtrate was evaporated. The latter yielded 58 g. of a dark brown oil which was chromatographed on three columns (35 × 4·5 cm.) of alumina from light petroleum (boiling-point 40° to 60° C.). Development with 5 litres of the same solvent gave the following chromatogram, as seen in ultra-violet light, the figures on the left giving thicknesses in millimetres :

Top : 25 orange, Fraction A, containing a carotenoid

$60\left\{\begin{matrix}\text{yellow}\\ \text{sandy}\end{matrix}\right\}$ Fraction B

$140\left\{\begin{matrix}\text{white, broad}\\ \text{orange-yellow}\end{matrix}\right\}$ Fraction C

Bottom : blue fluorescence. This with the filtrate gave Fraction D

Elution was accomplished with a mixture (8 : 1 : 1) of benzene, acetone and methyl alcohol. For the isolation of β-tocopherol only Fractions B and C need to be considered. These weighed respectively 14·7 g. and 25·5 g.

Fraction B : The solution in 100 ml. of methyl alcohol deposited 1·4 g. of sterols on standing for 24 hours. The oily residue left on evaporation of the filtrate was dissolved in 250 ml. of 90 per cent ethyl alcohol and the tritisterols (Karrer and Salomon 1) were removed by precipitation with digitonin (14 g.). The residue obtained after vacuum distillation was dissolved in benzene, and the solution was filtered from digitonin and evaporated. The oil so obtained (9·5 g.) was active in a dose of 15 mg.

Its solution in about 250 ml. of dry benzene was saturated with cyanic acid (prepared from 15 g. of cyanuric acid by heating slowly in a current of carbon dioxide) at 0° to 5° C., and filtered after standing for 48 hours at 5° C. The residue of cyamelide was washed repeatedly with hot benzene. The combined filtrate and washings were distilled, the residue was dissolved in 100 ml. of cold acetone, and the solution was filtered from insoluble material and evaporated. The residual oil was dissolved in petroleum (boiling-point 60° to 80° C.) and the solution was chromatographed on alumina. The difficulty encountered by the separation of a solid substance on the column was overcome by first shaking the solution with 30 g.

of alumina (Fraction 1) and then pouring the filtrate on to the column (30 × 2 cm.). The following chromatogram was obtained :

Top :	120 pale brown 30 orange	divided into two equal parts, Fractions 2 and 3 respectively
Bottom :	120 colourless,	Fraction 4, rejected

Each portion was eluted with a mixture (8 : 1 : 1) of benzene, acetone and methyl alcohol. The methyl alcohol solution (50 ml.) of Fraction 1 deposited a brown solid on standing in the ice-chest, and this was filtered off and stirred with cold acetone. The filtrate was evaporated and the residue was dissolved in warm methyl alcohol. After filtering off a trace of α-tocopheryl allophanate (?), β-tocopheryl allophanate (550 mg.) separated out as yellow crystals, melting-point 137° to 142° C. A further 130 mg. were obtained in a similar way from Fraction 2. The combined uncrystallisable residues from Fractions 1 and 2 were dissolved in petroleum (boiling-point 100° to 120° C.) and chromatographed as before. The column had an appearance similar to that described above. The lower half of the pale brown zone (120 mm.) yielded 250 mg. of the β-allophanate, melting-point 134° to 137° C.

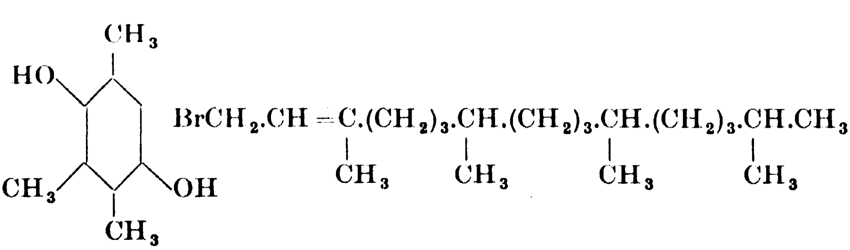
Fraction C: The oil (25·5 g.) was dissolved in light petroleum (boiling-point 40° to 60° C.) and chromatographed on a column (40 × 5 cm.) of alumina. On developing with 3 litres of solvent, it gave an ultra-chromatogram comprising 7 zones. The column was divided up as follows : (*a*) 80 mm. yellowish, (*b*) 70 mm. orange, (*c*) 170 mm. orange and (*d*) 70 mm. orange. The oil obtained from zone (*b*) deposited sterols on standing in methyl alcohol solution, and the filtrate was freed from tritisterols by treatment with 4 g. of digitonin. The resulting oil (3·5 g.) was then treated with cyanic acid and the mixture of allophanates so obtained was chromatographed. The topmost zone yielded only β-tocopheryl allophanate, 150 mg. being obtained ; melting-point 137° to 142° C.

Total yield : From 250 g. of starting material, 1·08 g. of β-tocopheryl allophanate were obtained, together with 1 g. of allophanate of melting-point 250° C. and 0·7 g. of allophanate of melting-point 70° C.

(*d*) **Purification of β-Tocopherol (Cumotocopherol)** by way of its *p*-nitrobenzoate (John 2): The allophanate

(125 mg.) was saponified by boiling for half an hour with 10 per cent methyl alcoholic potassium hydroxide solution in an atmosphere of nitrogen ; the oily tocopherol that resulted was carefully dried, allowed to stand with 1·5 moles of *p*-nitrobenzoyl chloride in absolute pyridine for 24 hours and then warmed on a water-bath for half an hour. The resulting yellow oil was dissolved in dry benzene, and the solution was poured on to a column of alumina. Two zones were formed, the upper narrow and dark yellow in colour formed by impurities, the lower broad and bright yellow in colour. The latter, which contained 130 mg. of substance, was washed through into the filtrate. This was evaporated and the acetone solution of the residue was treated with absolute alcohol ; on standing in the ice-chest, the solution deposited a lemon-yellow powder in the form of feathery crystals which had an unsharp melting-point at 38° to 40° C. (constant). This substance could be reconverted into an allophanate with properties identical with those of the original substance.

(*e*) **Condensation Product of Trimethylhydroquinone and Phytyl Bromide, presumably Racemic α-Tocopherol** (Karrer, Fritzsche, Ringier and Salomon) : The synthesis of the tocopherol molecule was accomplished by treating a solution of 1·7 g. of trimethyl hydroquinone in 10 ml. of dry petroleum (boiling-point 80° to 100° C.) with 1 g. of anhydrous zinc chloride and 4·8 g. of phytyl bromide and warming the mixture at 60° to 70° C. in a current of nitrogen. The rapid evolution of hydrogen bromide lasted about half an hour, but the mixture was heated for a further 1½ hours ; water was then added, and the petroleum layer was washed with dilute alkali solution and water, dried and chromatographed on alumina. The column, on being developed, was grey in colour, except for a small yellowish zone at the bottom. The main grey zone was eluted



Trimethyl hydroquinone Phytyl bromide

with a mixture of methyl alcohol and ether, the eluate was evaporated and the fractionation was repeated. The second column had a small zone at the top which was separated and eluted, giving a bright yellow oil, $C_{29}H_{50}O_2$. The yield was almost quantitative and the substance gave an allophanate melting-point 172° C.

Vitamin K

Dam and Schönheyder have investigated the concentration and also the isolation of this anti-hæmorrhagic factor from alfalfa. Powdered sugar was used as the adsorbent in the chromatograph ; after the pigments present had been washed through the column with petroleum, the sugar was dissolved in water and the substance was extracted with light petroleum. At first it seemed probable that calcium carbonate was a suitable adsorbent, but attempts to reproduce the earlier results proved abortive. It was found that the vitamin was destroyed by adsorbing it from petroleum solution on very active materials such as alumina or magnesium oxide (Dam and Lewis).

11. Hormones

Adrenaline

Concentration of Adrenaline (Fink 2)

The hormone is quantitatively adsorbed when its solution (*p*H 7·2 to 7·3) in hydrochloric acid, containing 0·15 g. per litre, is filtered through a mixture of asbestos and kaolin. The adrenaline is dissolved from the filter mass by hydrochloric acid.

Estimation of Adrenaline in Blood (Whitehorn)

Fresh blood (7 to 10 ml.) was poured without delay into 10 volumes of 3 per cent trichloracetic acid and allowed to stand for from 15 minutes to several hours. The mixture was then filtered or centrifuged and the clear liquor was drawn through a column (20 × 2 cm.) of pure silica (*Acidum silicum*) previously boiled for a quarter of an hour with 5 per cent sulphuric acid, and then washed by decantation 10 or 12 times with water.

The column was washed first with water and then with 1 to 3 ml. of sodium bisulphite solution (5 g. of the anhydrous salt in 50 ml. of water) until the filtrate reacted neutral or alkaline to bromthymol blue. Alternatively, the filtrate from the trichlor-acetic acid precipitation (corresponding to 5 ml. of blood) was made approximately neutral to bromthymol blue with *N*-sodium hydroxide solution and then treated with 1 ml. of buffer solution (containing 17·4 g. of dipotassium hydrogen phosphate and 6·8 g. of potassium dihydrogen phosphate in 100 ml.). The mixture was filtered and then drawn through the adsorbent at the rate of 3 drops per second. The column was developed with three 8-ml. portions of freshly boiled distilled water. Under these conditions, adrenaline (epinephrine) is adsorbed, whilst glutathione and other non-basic or weakly basic substances pass into the filtrate. The adrenaline was eluted with 5 ml. of 2/3 *N*-sulphuric acid and the amount was estimated according to established methods.

Sex Hormones and Related Substances

Isolation from Mare's Urine

(*a*) ŒSTRONE AND EQUILENIN (Duschinsky and Lederer). Œstrone: The acidified urine from pregnant mares was extracted with benzene and the hormone was concentrated by partitioning between aqueous alcohol and light petroleum; the active substance was present in the lower layer. Five grams of the dark resinous concentrate thus obtained, which contained about 3,750,000 I.U. per g., were dissolved in 250 ml. of benzene and the solution was poured on to a column of calcium hydroxide. The tube used measured 25 × 4·5 cm. and was closed at the lower end by a Jena sintered-glass plate. Part of the pigments remained adsorbed at the top of the column as a brown zone, whilst the remainder slowly travelled downwards as a reddish-violet band which served as an indicator, for the œstrone-rich zone invariably occurred immediately above this ring, sometimes extending into the coloured region. After developing with 175 ml. of benzene, the coloured ring was almost at the bottom of the column, and the chromatogram had the following appearance:

Top :	10 dark brown	
	25 yellow	
	50 white	
	55 white	containing half the total hormone
	60 violet-red	containing half the total hormone
Bottom :	30 yellow	

The œstrone-rich zone was eluted with acetone, or, alternatively, the lime was dissolved in hydrochloric acid and the solution was extracted with ether and alcohol. The resulting material (140 mg.) contained 6,500,000 I.U. per g., and from it pure colourless œstrone, $C_{18}H_{22}O_2$, was obtained on recrystallisation from alcohol. It had melting-point 258° C. and contained 10,000,000 I.U. per g.

Equilenin : A more satisfactory chromatogram was formed when a mixture (10 : 1) of alumina (Brockmann) and fibrous alumina was used instead of lime. The reddish-violet zone formed at the top of the column was eluted with acetone, and the eluate was recrystallised from cyclohexane, giving reddish-brown needles of indirubin,[1] $C_{16}H_{10}O_2N_2$, melting-point 315° C. Immediately below this pigment band was the hormone-rich zone. This gave on elution a product, 45 mg. of which were dissolved in 2 ml. of alcohol ; the solution was treated with 2·5 ml. of 10 per cent alcoholic picric acid solution. In a short time equilenin picrate crystallised out in the form of red needles, melting-point 204° to 206° C. The free equilenin formed white needles, melting-point 256° to 258° C.

(*b*) The application of chromatography to the preparation of the international standard of œstrone is recommended in a patent specification of Hoffmann-La Roche A.G., but no details are given concerning the procedure to be followed. The starting material comprised 100 litres of stallion's urine, which was extracted several times with benzene, after being subjected to acid hydrolysis. The partially concentrated extract was washed with sodium hydroxide solution and evaporated, and

[1] Indirubin was isolated by Musajo from the urine of rats fed on a protein-rich diet. Ten litres of the alkaline urine were extracted in 1-litre portions with toluene. The deep red extract gave 4 zones when chromatographed on alumina ; the column was developed with benzene and the zones were, respectively, yellowish-brown, reddish-violet, ash-grey and bright grey. The second of these was eluted with acetone and the residue left after distilling off the solvent was taken up in ether. On standing, fine needles deposited, which, after recrystallisation from cyclohexane, had melting-point over 340° C. Yield, 11 mg.

the residue was repeatedly hydrolysed with hydrochloric acid *and then steam-distilled.* The residual resin was dissolved in ether and the solution was shaken several times with sodium hydroxide solution. Œstrone was then extracted from the alkaline layer with benzene, evaporation of the extract giving 1 g. of œstrone of 90 per cent purity. A purer product was obtained by means of chromatography. The pure œstrone had melting-point 259° to 260° C. and $[\alpha]_D$ (chloroform) $= + 160°$.

(*c*) 17-DIHYDROEQUILENIN, β-ŒSTRADIOL AND " COMPOUND 3 " (Hirschmann and Wintersteiner) : A crude preparation of β-œstradiol was obtained from the urine of pregnant mares. Though free from dihydroequilenin, this material contained about 6 per cent of " compound 3," which could only be removed by adsorption analysis. These workers followed the procedure adopted by Duschinsky and Lederer. A solution of 67 mg. of the material in 45 ml. of benzene was filtered slowly through a column (12·5 × 0·6 cm.) of alumina (Brockmann) and this was developed with large volumes of benzene. Unsaturated impurities, such as " compound 3," were adsorbed at the top, whilst β-œstradiol was retained lower down the column. The orientation of the zones was determined with the aid of a colour reaction, using *p*-nitrodiazobenzene. A little of the adsorbent was extracted with a few drops of alcohol and the reagent was added to the solution. " Compound 3 " gave a yellow colour, whereas β-œstradiol and dihydroequilenin gave, respectively, yellow and red colours only after their solutions had been made alkaline with sodium hydroxide solution and then acidified with acetic acid. The œstradiol was eluted by extraction in a Soxhlet apparatus with 95 per cent alcohol, a yield of 48 mg. being obtained. Preparations consisting mainly of " compound 3," $C_{18}H_{22}O_2$, together with a small amount of dihydroequilenin, were split into their individual components by a similar method, using repeated adsorption on columns of alumina and a 30 : 1 mixture of benzene and acetone for development. The colour reaction referred to above was utilised to fix the position of the zones.

Conversion Products of Sex Hormones

(*a*) DISPROPORTIONATION OF DIHYDROEQUILIN (Serini and Logemann 1) : When a solution of 0·5 g. of dihydroequilin,

$C_{18}H_{22}O_2$, in 30 ml. of methyl alcohol was shaken with Raney nickel in an atmosphere of hydrogen for 1 hour, no hydrogen was taken up, but a mixture of the dehydrogenation product, dihydroequilenin, $C_{18}H_{20}O_2$, and the hydrogenation product, iso-œstradiol, $C_{18}H_{24}O_2$, was obtained. The residue left after evaporation of the filtered solution was dissolved in a mixture of benzene and ether and the solution was poured on to a column of alumina (Brockmann). The column was washed first with a mixture (5 : 1) of benzene and acetone and the filtrate was evaporated. Repeated recrystallisation from dilute methyl alcohol and then from chloroform yielded pure iso-œstradiol, melting-point 181° C. The column was then washed with methyl alcohol and the filtrate from this washing yielded dihydroequilenin, which after recrystallisation from methyl alcohol had melting-point 245° C.

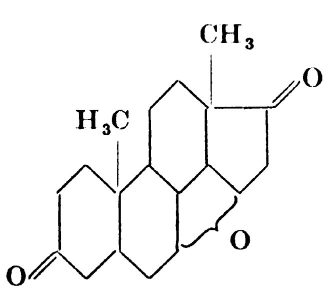
(*b*) PARTIAL HYDROGENATION OF PREGNANDIONE TO EPI-PREGNANOL-3-ONE-20 (Butenandt and Müller): Platinum black (1 g.) was suspended in ether and saturated with air for half an hour and then with hydrogen. A solution of 1 g. of pregnandione, $C_{21}H_{32}O_2$, in alcohol was added and the mixture was hydrogenated until one molecule of hydrogen had been taken up; the reaction product was crystallised from dilute methyl alcohol. From 5 experiments, in which a total of 4·8 g. of the dione was reduced, 510 mg. of sparingly-soluble *n*-pregnanol-3-one-20 were obtained, whilst 300 mg. of epi-pregnanol-3-one-20 were obtained by crystallisation from dilute acetone of the residue from the mother-liquors. The bulk of the hydrogenation product, about 4 g., remained behind in the form of a low-melting solid; it could not readily be separated into its components either by crystallisation or sublimation. Adsorption on alumina from a mixture (1 : 5) of acetone and light petroleum, however, yielded a further 1·32 g. of pure epi-pregnanolone, $C_{21}H_{34}O_2$, as the readily adsorbable fraction. It had melting-point 148° to 149° C.

(*c*) 20 : 21-MONOACETONE-Δ^4-PREGNENDIOL (20, 21)-ONE-3, $C_{24}H_{36}O_3$, FROM MONO-ACETONE-PREGNENTRIOL (Steiger and Reichstein 2): The oil (0·39 g.) obtained by high vacuum distillation of the product of dehydrogenation of 0·375 g. of triol with aluminium tertiary butylate was dissolved in 5 ml. of pentane and the solution was filtered through a column (5 g.)

of alumina already saturated with pentane. It was washed twice with 10-ml. portions of pentane and the filtrates were rejected, as was a third filtrate obtained by washing the column with a 1 : 4 mixture of benzene and pentane. Finally the column was washed with a 1 : 1 mixture of benzene and pentane until the filtrate no longer gave a residue on evaporation. The residue obtained from this fraction crystallised on standing with a little pentane ; the substance was recrystallised from pentane and yielded the α-form of the oxidation product. The lower melting fractions and resinous residues were re-chromatographed on alumina (4 g.) and, as before, all the material was washed off the column by a 1 : 1 mixture of benzene and pentane. In all, 245 mg. of a substance of melting-point 124° to 125° C. were obtained. The mother-liquors from two such experiments were again treated with aluminium tertiary butylate. The product obtained by an analogous chromatographic adsorption was the pure β-form of the oxidation product and had melting-point 132° C.

$\Delta^{5:6}$-Pregnen-tetrol-(3 : 17 : 20 : 21)-triacetate-(3 : 20 : 21) was purified by adsorption on alumina from benzene solution (Serini and Logemann 2). When the column was eluted fractionally with a 20 : 1 mixture of benzene and acetone, the first fraction was found to contain most of the product. It was concentrated, and crystallised on standing. After recrystallising from a mixture of benzene and light petroleum, it had melting-point 166° to 167° C.

(*d*) The crude substance obtained by Steiger and Reichstein (1) by hydrogenation of 40 mg. of slightly impure andrenosterone melted between 170° and 182° C., and was freed from impurities by the following procedure.



Hydrogenation product of andrenosterone

It was dissolved in a little pure benzene and the solution was diluted with pentane and poured on to a column (1·6 × 0·8 cm.) of alumina (Brockmann) saturated with pentane. The filtrate yielded 8 mg. of a syrupy substance on evaporation, whilst most of the hydrogenation product was removed from the column by treatment with absolute ether. Evaporation and the addition of a little pentane to the residue yielded 15 mg. of crystalline triketone, $C_{19}H_{26}O_3$, in the form of leaflets of melting-point 178° to 180° C. (corr.).

(*e*) PREPARATION OF 17-VINYL-TESTOSTERONE (Ruzicka, Hofmann and Mehldahl). A solution of 200 mg. of Δ^5-17-vinyl-3 trans-17-dihydroxy-androstene in 10 ml. of dry acetone was heated under reflux for 20 hours with a solution of 400 mg. of aluminium tertiary butylate in 10 ml. of dry benzene. The acetone was removed as completely as possible by evaporation under reduced pressure, and the residue was taken up in ether and washed several times with dilute sulphuric acid and then with water. The ethereal solution, dried over sodium sulphate and distilled under reduced pressure, gave a colourless oil (250 mg.) containing admixed crystals. This was dissolved in 20 ml. of benzene and poured on to a column of alumina (5 g.) saturated with benzene. On washing with two 10-ml. portions of benzene, only a trace of oil was obtained from the filtrate. The eluate obtained on washing with a 2·5 : 1 mixture of benzene and ether, however, gave 130 mg. of oil on evaporation, and this crystallised from ether. Continued washing of the column with ether yielded a further 60 mg. of crystals. The combined material was recrystallised from a mixture of ether and pentane. The 17-vinyl-testosterone, $C_{21}H_{30}O_2$, so obtained formed needles of melting-point 140° to 141° C. The purification of 17-ethyl-testosterone, $C_{21}H_{32}O_2$, was carried out in a similar manner.

Fractionation of Several Constituents of the Adrenal Cortex (Steiger and Reichstein 3)

In the course of the investigations into the constituents, active and inactive, of the adrenal cortex, carried out by Reichstein and his school, a crude crystallisate was obtained at one stage and consisted of a mixture of polyvalent alcohols of the allo-pregnane series. Fractional crystallisation being use-

less, the mixture was acetylated and the product was repeatedly chromatographed. In this way, four individual substances were ultimately isolated. It is impossible here to give a complete account of the experiments of Steiger and Reichstein (3), but the following extract affords a beautiful example of the technique of "liquid chromatography."

"CRYSTAL FRACTION B." Three hundred and fifty milligrams were acetylated by standing for 30 hours with 6 ml. of pyridine and 5 ml. of acetic anhydride. The residue obtained by evaporating under reduced pressure was dissolved in ether and the solution was washed successively with dilute hydrochloric acid, sodium hydroxide solution and water ; it was then dried and evaporated. The residue was sublimed at a temperature of 155° C. in a high vacuum (0·001 mm.) and the sublimate was dissolved in a little benzene. The solution was diluted with 10 volumes of pentane and poured on to a column of alumina (12 g.) saturated with pentane. The following table indicates the "liquid chromatogram" fractions that resulted :

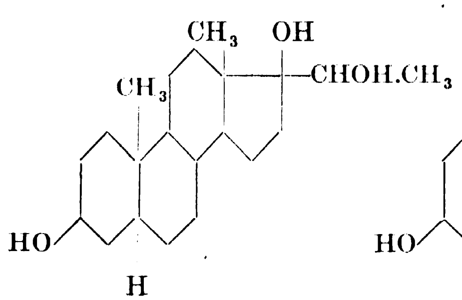
TABLE 20

SEPARATION OF ALLO-PREGNANTRIOL-(3 : 17 : 20) AND ALLO-PREGNANTETROL-(3 : 17 : 20 : 21) BY MEANS OF A "LIQUID CHROMATOGRAM" (Steiger and Reichstein (3))

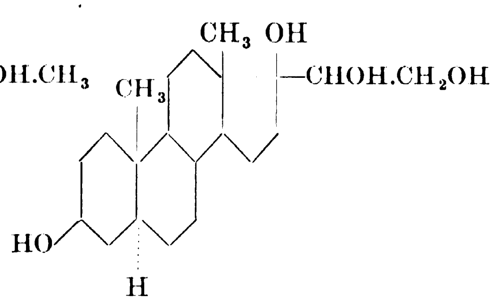
Fraction	Developed with		Nature of Residue left on Evaporation, after Treatment with Ether-Pentane Mixture
1 . .	40 ml. pentane		Trace of oil
2 . .	20 ml. benzene-pentane	(1 : 4)	Trace of crystals
3 . .	,, ,,	,,	,, ,,
4 . .	,, ,,	(1 : 1)	Crystals, m.p. 160° C.
5 . .	,, ,,	,,	,, ,, 161° C.
6 . .	,, ,,	,,	,, ,, ,,
7 . .	,, ,,	,,	,, ,, ,,
8 . .	,, ,,	,,	,, ,, ,,
9 . .	,, benzene		,, ,, ,,
10 . .	40 ml. ,,		,, ,, ,,
11 . .	60 ml. ,,		,, ,, ,, (trace)
12 . .	40 ml. ether		,, ,, 175° C.
13 . .	,, ,,		,, ,, 174° C.
14 . .	60 ml. ,,		,, ,, 172° C. (trace)
15 . .	40 ml. acetone		,, ,, 154° to 175° C.
16 . .	,, ,,		,, (trace)
17 . .	20 ml. ,,		None

Fractions 2 to 11 gave on recrystallisation from a mixture of ether and pentane 205 mg. of the pure diacetate of "sub-

stance J" of melting-point 161° to 162° C. (corr.), whilst fractions 12 to 14 gave 57 mg. of the pure triacetate of "substance K," melting-point 177° C.



Allopregnantriol-(3 : 17 : 20)
"Substance J"

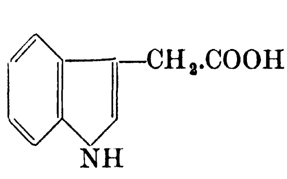


Allopregnantetrol-(3 : 17 : 20 : 21)
"Substance K"

Auxins

Purification of Hetero-auxin (Kögl, Haagen-Smit and Erxleben)

Hetero-auxin, the plant-hormone, isolated by these workers from urine, appeared to be identical in all respects with synthetic β-indolyl-acetic acid, $C_{10}H_9O_2N$, except that the natural material exhibited optical rotation. Thus, assuming the two substances to be identical, the natural material was evidently contaminated with a lævo-rotatory impurity. The colour reaction with ferric chloride-hydrochloric acid, together with the measurement of rotation, facilitated the purification of the hormone.



Hetero-auxin

A solution of 50 mg. of hetero-auxin (from urine) in 50 ml. of benzene was drawn through a column (40 × 4·5 cm.) of calcium carbonate, which was developed first with 50 ml. of benzene, then with 30 ml. of a 10 : 1 mixture of benzene and alcohol, and finally with 30 ml. of a 2 : 1 mixture of benzene and alcohol. The column was then sucked as dry as possible

and cut into six quite arbitrary portions, each of which was eluted by extracting three times with cold alcohol. The alcohol extracts were evaporated with the following results :

Top :	(*a*)	2·9 mg., colour reaction negative
	(*b*)	9 mg., weak colour reaction, no rotation
	(*c*)	18 mg., strong colour reaction, no rotation
	(*d*)	14 mg., distinct colour reaction, no rotation
	(*e*)	3 mg., weak colour reaction
Bottom :	(*f*)	no appreciable residue

A second adsorption with more material was carried out and the residues from fractions (*b*) to (*e*) in both experiments were combined and recrystallised from water. A yield of 89 mg. of pure optically inactive hetero-auxin, melting-point 165° C., was obtained. Fractions (*a*) from the two chromatograms were combined and yielded 6·1 mg. of an unidentified lævo-rotatory substance, $[\alpha]_D^{20} = -34{\cdot}3°$ (in alcohol) ; melting-point 167° C. It was inactive when tested by Went's method.

12. Enzymes, Co-enzymes and Biochemical Activators

For the early history of enzyme adsorption, see Willstätter (2). Chromatographic analysis has been used only to a limited extent for the purification of substances of this type, but the experiments of Willstätter on the adsorption and elution of enzymes indicate that the method has interesting possibilities. It remains to be seen how far the special advantages of the Tswett method can be made use of with such substances. Preliminary experiments recorded in the literature suggest important developments in the near future.

Adler and Michaelis did not succeed in separating dehydrase from flavin-enzyme on a column of aluminium hydroxide, since both substances distributed themselves over the whole of the column, although the lower portion contained less of the flavin and more of the dehydrase.

Separation of Co-zymase and Co-dehydrase II (Euler and Adler)

These workers found two co-dehydrases in yeast. One, co-zymase, is an essential component of the yeast alcohol-dehydrogenation system, but is inactive in Robison's ester-

dehydrogenation system ; whilst the other, co-dehydrase II, has the opposite action. The two co-enzymes were separated from one another by chromatographing an aqueous solution on alumina (Brockmann), the co-dehydrase II being adsorbed in the upper part of the column, whilst the co-zymase passed into the filtrate on developing the column. The method of dividing up the column was a purely empirical one, and the corresponding eluates were tested for their activity in the Robison ester-dehydrogenation system, in the alcohol-dehydrogenation system and in alcoholic fermentation. The ultra-chromatogram is of no value because fluorescent impurities are present.

A filtered solution of 100 mg. of an average preparation (obtained from the copper precipitation mother-liquors of a co-enzyme preparation) in 10 ml. of water was poured on to a column (8 × 0·7 cm.) of alumina prepared from an aqueous suspension. The column was first washed with 15 ml. of water, with the application of gentle suction, giving 21 ml. of filtrate. The column was then washed with 100 ml. of water which yielded 94 ml. of filtrate, the filtration occupying 2 hours. The upper fluorescent zone of the column was divided into small portions, and the remainder of the column into larger portions ; these were dried *in vacuo* and weighed. The co-enzyme content of each of the adsorbates was tested by the fermentation method, and each was tested in addition for its activity in the alcohol-dehydrogenation and Robison's ester-dehydrogenation systems (Thunberg's method). A very satisfactory fractionation was obtained, for of 2,260 co-zymase units present in the original preparation, 1,890 were recovered in the filtrate, and of the 452 co-dehydrase II units originally present, 294, 140 and 18 respectively were recovered from the three upper zones of the adsorbent. The rest of the column and the two filtrates were free from co-dehydrase II.

Co-dehydrase II is also present in ox-liver (Das). A solution (10 ml.) of Harrison's co-enzyme preparation, corresponding to 50 g. of acetone-dried liver, was poured on to Brockmann alumina (5 × 0·7 cm.) and the column was washed with 50 ml. of water. It was then cut up into 5 equal portions. The top three yielded co-dehydrase II, but were free from co-zymase. The method of testing the fractions was similar to that described above.

Preparation of Pure Co-zymase from Yeast (Euler and Schlenk 2)

The chromatographic method of isolating the co-enzyme can be employed even when a high degree of purity is required and when co-zymase has to be prepared on a relatively large scale. Simply shaking a solution with adsorbent does not, however, give the same result as is obtained by means of a column, for only a portion of the co-enzyme is removed on eluting, and, moreover, impurities are invariably eluted at the same time. Nor does the use of different degrees of acidity for the elution affect this result.

The troublesome purification which must precede the chromatographic adsorption cannot be described here. A solution of 1 g. of co-zymase preparation (ACo = 400,000) in 25 ml. of water was centrifuged, if necessary, to remove insoluble matter, and the solution was then treated with saturated baryta solution with cooling and stirring until the *p*H was 6·5. After 10 minutes, the flocculent precipitate was centrifuged off and washed with 5 ml. of 10 per cent barium acetate solution. The barium was removed from the combined centrifugate and washings by the dropwise addition of ice-cold 0·5 *N*-sulphuric acid, the solution being kept cold all the time. The barium sulphate was removed and washed with 5 ml. of water, and the washings were added to the filtrate.

The bottom end of a glass tube, 7 to 8 mm. internal diameter, was opened out so as to form a lip over which a piece of soft copper gauze was fastened with wire. A plug of damp cotton-wool was forced into the tube against the gauze, and the tube was then fitted into a filter flask and filled up with water. When most of the water had run out and the level had almost reached the cotton-wool, a freshly prepared suspension of Brockmann alumina was poured into the tube, until a column of adsorbent 9 to 10 cm. high had been formed. In this way the formation of air-bubbles was avoided, and the alumina was packed uniformly. So far no suction had been used. When almost all the water had filtered through, the receiver was quickly emptied and the co-zymase solution was poured on to the column. Gentle suction was applied when filtration became difficult. Each 10-ml. portion of the filtrate was stored separ-

ately in the ice-chest and the fermentation test was applied as soon as convenient. The first 20 ml. were almost inactive, but the co-enzyme content increased suddenly, and most of it came through in the next portion of the filtrate. Meanwhile water was continuously added to the column, which was not allowed to become dry. After washing with about 100 ml. of water, the rest of the filtrate contained such a small amount of co-zymase, as indicated by the yeast test, that it was rejected. The time required for the solution to filter through the column was under half an hour, whilst the washing occupied about an hour. The suction had to be increased gradually during the operation. Since each fermentation test occupies from 2 to 3 hours, it is advisable in all instances to wash with an excess of water. The fractions with an activity of more than 100 ACo per ml. were combined, the total volume being 80 to 100 ml. A slight turbidity was removed by centrifuging and the solution was then precipitated by the gradual addition of 10 volumes of ice-cold alcohol. For details of the subsequent operations the original paper should be consulted. A yield of 300 to 400 mg. of material ACo = 600,000 to 630,000 was obtained.

The preparation thus obtained by adsorption contained aluminium, which was removed by adjusting the *p*H to 7·0 with 0·5 *N* ammonia, and filtering off the precipitate of aluminium hydroxide.

Chromatographic Separation of some of the Enzymes of Emulsin

According to some recent experiments by Zechmeister, Tóth and Bálint, aqueous solutions of emulsin Merck were chromatographed on a column of bauxite in the following way. Under certain conditions (acetate buffer solution at *p*H 4·7) β-glucosidase is easily and completely adsorbed, being eluted subsequently by 0·1 per cent ammonia. The α-galactosidase and chitinase (substrates, raffinose and chitodextrin respectively) on the other hand ran through into the filtrate. The stability of the accompanying substances in the solution was so altered by this treatment that in a second analogous experiment the α-galactosidase was also adsorbed and so separated from chitinase. Elution was brought about as before. Other experiments directed at the separation of mixtures of enzymes

by chromatography are being undertaken by Zechmeister and his collaborators.[1]

Inhibition of Blood Coagulation (Chargaff 3)

A mixture of 3·5 litres of sheep's blood and a solution of 9·4 g. of sodium oxalate in 1·2 litres of isotonic sodium chloride solution was centrifuged for 30 minutes. The sedimented blood-cells were washed several times in the cold with isotonic salt solution containing 0·1 per cent of sodium oxalate, and finally with salt solution only. The blood-cells were extracted successively with acetone, ether, a 3 : 1 mixture of methyl alcohol and chloroform, and two 1-litre portions of boiling absolute alcohol. There was thus obtained 0·46 g. of a brown powder. When this was treated with warm glacial acetic acid, a small amount of material remained insoluble and the solution yielded about 0·2 g. of active material soluble in a 1 : 3 mixture of chloroform and ethyl acetate. A solution of this active fraction in a 9 : 1 mixture of methyl alcohol and petroleum was filtered slowly through a column of alumina (Brockmann); coloured impurities were adsorbed whilst the inhibitor itself passed through into the filtrate.

[1] See *Enzymologia*, **5**, 302 (1938); **7**, 165, 170 (1939).—TRANSLATORS.

CHAPTER 6

APPLICATIONS TO TECHNOLOGY

1. Technical Tannin-extracts

The chromatography of such solutions was described by Grassmann and by Grassmann and Lang, who used this method for the characterisation and differentiation of the individual components of these complex mixtures. In the same way, it is possible to obtain some information concerning the nature of the pigments present in the extractable constituents of leather. In such instances, fluorescence chromatography gives the most satisfactory results.

Simple aqueous solutions can seldom be employed. Thus, for example, 3 ml. of a concentrated aqueous extract (containing 20 to 30 per cent of total solids) is treated with 2 volumes of methyl alcohol and any precipitate that forms is filtered or centrifuged off. Using the apparatus described on page 82, the solution is then chromatographed on alumina (or " tonsil ") or magnesium oxide, the column being prepared dry and then saturated with 2 ml. of methyl alcohol just before addition of the dye solution. The column is washed with 3 to 4 ml. of ethyl acetate or with a similar volume of a mixture (1 : 3) of methyl alcohol and ethyl acetate. The chromatogram is examined in ultra-violet light.

PINE-BARK EXTRACT, chromatographed on alumina, formed a dark greyish-brown region at the top of the column, with a bright yellowish-green zone below it, then a narrow strongly fluorescent band with a rather broader blue fluorescent zone immediately below this. An acid reaction increased the intensity of the fluorescence of the whole chromatogram, whilst an alkaline reaction had the opposite effect. At pH 2·2 a pale apple-green colour appeared at the top of the column, and a pale blue colour at pH 7·7.

OAK-WOOD EXTRACT, also chromatographed on alumina, gave a similar broad zone, dark brown in colour and non-fluorescent, with a bright greenish-blue zone below. Usually these two zones were separated by a narrow yellowish-green ring.

QUEBRACHO-WOOD EXTRACT was chromatographed in neutral or feebly acid solution on alumina. At the top of the column was a coloured zone with a lemon-yellow or ochre-yellow fluorescence, and below a green or steel-blue zone, the two zones being separated by a broad black or dark brown band. With strongly acid solutions, the first zone had a yellowish-green fluorescence and the middle dark-coloured zone a sienna-brown or greenish-brown fluorescence. Usually this was succeeded by a new zone coloured green or greyish-blue.

DIFFERENTIATION OF CHESTNUT- AND OAK-WOOD EXTRACTS : The extract (containing 20 to 30 per cent of total solids) is treated with bromine water until a faint smell of bromine persists, and the mixture is then warmed on the steam-bath until the smell disappears ; two volumes of methyl alcohol are then added and the solution is filtered. Although a less intense chromatogram is obtained after such treatment, that from the oak extract gives a bluish-green fluorescent zone, easily distinguishable from the pure blue fluorescent zone given by a chestnut extract.

TANNIN (" zur Analyse, Merck ") gave a uniform indigo-blue fluorescence when a methyl alcohol solution was chromatographed on alumina or magnesium oxide.

CATECHIN from gambier, several times recrystallised, gave a green fluorescence on alumina and a pale steel-blue fluorescence on magnesium oxide.

TANIGAN O (synthetic dye) gave the following zones when chromatographed on alumina : black, sulphur-yellow, dark brown, reddish-brown, sulphur-yellow, bright yellowish-green, bright violet.

Further experiments are described in the original papers.

2. Technical Oils and Fats. Butter

The adsorption technique is well adapted to the characterisation of commercial oils and for the detection of added dyes.

According to Boekenoogen (1, 2 and private communication), many vegetable oils can be poured on to a column of alumina (Merck) without previous dilution, and the column can then be developed with light petroleum or carbon disulphide. With some oils, such as linseed, that yield complex chromatograms, the lower half of the column is composed of a mixture of alumina with frankonite H, so as to adsorb all the pigments present. In general, natural oils yield complex chromatograms, whereas oils that have been bleached by some kind of adsorption process and have thereby lost the greater part of their pigment content, yield quite simple chromatograms. Chlorophyll, or a derivative, is frequently present as a constituent of several commercial oils, in which it can be detected by means of its absorption spectrum, e.g., in cottonseed oil, palm oil, sunflower-seed oil and tea-seed oil. Green pigments are present in even larger quantities in linseed oil, olive oil, rape oil, mustard oil and soya-bean oil. Occasionally also pigments are observed that have not yet been identified. (Cf. Table 21.)

TABLE 21

BEHAVIOUR OF SOME OILS ON ALUMINA, AFTER DEVELOPING WITH PETROLEUM OR CARBON DISULPHIDE (Boekenoogen 1)

Oil	Chromatogram	Filtrate
Arachis	Yellow zone, travelling slowly down the column	Colourless
Sesame	Violet-red above, brownish-yellow below	Pale yellow
Palm	Surface green, brownish-yellow below, the rest of the column pink	Orange red
Olive	Surface green and brown, no definite chromatogram	Yellow
Sunflower	Green, brownish-yellow zone below	Yellow
Linseed	Surface green, then a broad yellow zone, becoming greenish lower down with a faintly coloured region below	Yellow
Rape	Similar to linseed	Yellow

The detection of added colouring-matters was studied by Boekenoogen and by Thaler (1, 2). Alumina (Brockmann) and bleaching earth ("Clarit") were used as adsorbents, with benzene as solvent and developer (Table 22).

TABLE 22

DETECTION OF ADDED COLOURING-MATTERS (Thaler 1)
(10 g. of oil or cocoanut oil; benzene)

Colouring-matter	Alumina-column	Clarit-column
Carotene, pure	Pale yellow zone, yellow filtrate	Surface dark green when damp, greyish-blue when dry
Carotene, tech.	Surface bright yellow, orange below; yellow filtrate	Surface moss-green, grey-green below (when dry)
Carrot oil, tech.	Surface yellow, then narrow bright yellow band, rest of adsorbent pink	Surface dark green when damp, bluish-green when dry
Xanthophyll	Surface very pale reddish-grey	Surface grey when dry
Annatto in soya-oil	Surface deep orange-red	Surface bright grass-green, rest of column grey
Bixin, pure	Like annatto	Surface deep reddish-brown
Saffron extract (benzene)	Surface orange-yellow, bright yellow below	Surface dark yellowish-green (dry) shading to bright blue
Calendula extract (benzene)	Surface bright yellow, then yellow, then pink with 4 raspberry-red bands	Surface (dry) dark green, shading to narrow bright blue
Curcuma extract (benzene)	Surface orange red, with an orange-red zone below	Surface bluish-red when wet, bright brown when dry
Alkannin (tech.)	Surface violet, then pale yellow	Surface (dry) bright lilac
Dimethyl yellow	No adsorption	Surface (dry) blood red
p-Aminoazobenzene	Yellow ring, quickly washed through the column	Surface (dry) blood red
Martius' yellow	Surface orange-yellow	No adsorption
Sudan III	Red zone, quickly washed through the column; yellow and pink below	Broad violet band; rest of the column red
Scarlet R.	Reddish-brown slowly washed through the column	Broad sky-blue band and broad pink band
Fat Ponceau	Raspberry-red zone	Broad bright blue band

The method is very sensitive, depending to some extent, of course, on the nature of the pigment. Thus dimethyl yellow or *p*-aminoazobenzene can easily be detected in an amount of 0·01 mg. per 10 g. of fat; bixin can also be detected in very small amounts. In general, the acid dyes are readily adsorbed on the alumina and the basic on the bleaching earth. The examination of the clarit chromatogram is usually done after drying, because the adsorbent itself has a colour when moist.

Butter

The behaviour of summer butter on a column was investigated by Thaler (2). A filtered solution of 20 g. in

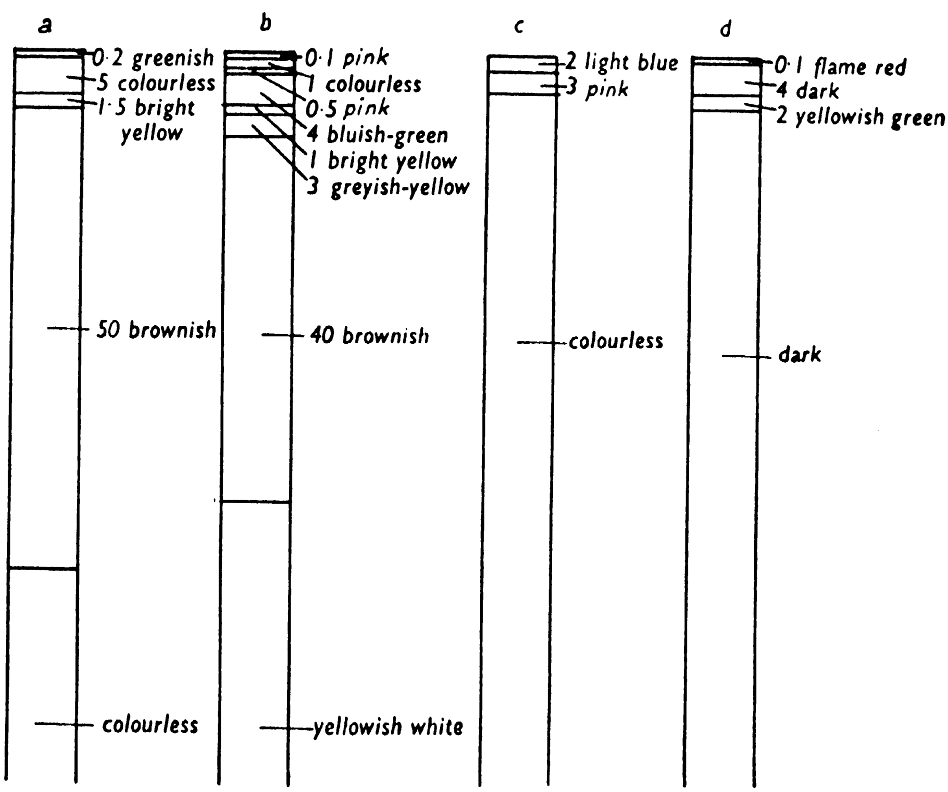


FIG. 39.—Chromatogram of uncoloured summer butter: (*a*) on alumina in daylight; (*b*) on alumina in ultra-violet light; (*c*) on clarit in daylight; (*d*) on clarit in ultra-violet light

50 ml. benzene at about 35° C. was poured on to a column of alumina or clarit, and developed with 20 to 30 ml. of benzene.

Natural butter produces a characteristic chromatogram with a few delicately coloured bands and with a characteristic appearance in ultra-violet light (Fig. 39). The presence of a small amount of added colouring matter gives rise to quite a different chromatogram with many vari-coloured foreign zones, the method thus affording a simple way of detecting sophistication, even if the butter has been coloured with carotenoids that normally occur in milk fat.

Investigation of the Unsaponifiable Matter of Oils and Fats

Selective adsorption has frequently been employed in investigations of this kind, usually from a solution in petroleum or petroleum-benzene mixture. Some of the relevant work has already been described (pp. 139, 141 *et seq.*; see also Zechmeister and Tuzson (4, 8) and the preparation of vitamin E concentrates from wheat-germ oil, p. 264). Large amounts of sterols interfere with the chromatography, so these must usually be removed beforehand either by freezing or by some other method. The rest of the unsaponifiable fraction is then retained at the top of the column.

Thorbjarnarson and Drummond isolated squalene, $C_{30}H_{50}$, in this way from the unsaponifiable fraction of olive oil, and Thorbjarnarson, Ruiz and Drummond also chromatographed the unsaponifiable matter of several fish oils, e.g., shark liver oil, herring oil and the liver oil of the rat-fish, *Chimæra monstrosa* (see also Drummond, Ruiz and Thorbjarnarson). Under the usual conditions, saturated and unsaturated hydrocarbons such as squalene and carotene run rapidly through the column, whilst saturated alcohols are adsorbed, generally at the top, with unsaturated alcohols below them, especially if much squalene is present. Sterols and polyene alcohols occupy a well-marked zone, for instance, in the upper third of the column.

$$CH_3-\underset{\underset{\displaystyle CH_3}{|}}{C}=CH.CH_2.CH_2.\underset{\underset{\displaystyle CH_3}{|}}{C}=CH.CH_2.CH_2.\underset{\underset{\displaystyle CH_3}{|}}{C}=CH.CH_2.CH_2.CH=\underset{\underset{\displaystyle CH_3}{|}}{C}.CH_2.CH_2.CH=\underset{\underset{\displaystyle CH_3}{|}}{C}.CH_2.CH_2.CH=\underset{\underset{\displaystyle CH_3}{|}}{C}-CH_3$$

Squalene

Example : The unsaponifiable matter from Greenland shark oil (sterol content 25 per cent) was freed from cholesterol and batyl alcohol, $C_{21}H_{42}O_3$, by cooling the methyl alcohol solution (and the resulting mother-liquors) to 0° C. The sterol-free residue, representing 6 per cent of the original oil, was dissolved in a little light petroleum (boiling-point 40° to 60° C.) and the solution was poured on to a column of alumina (Merck). After developing with the same solvent, the column was divided according to the zones formed, as follows :

Top :	Zone 1.	1·79 g.	Iodine No. 77·0	Pale yellow, viscous liquid
	„ 2.	2·05 g.	„ 72·3	Yellow, viscous liquid
	„ 3.	2·95 g.	„ 75·9	Dark-brown, semi-solid liquid
Bottom :	„ 4.	2·18 g.	„ 82·5	Yellowish, viscous oil
Filtrate :		1·74 g.	„ 152·5	Almost colourless, mobile liquid

The well-differentiated zones were eluted with a mixture of methyl alcohol and ether. Zone 1 yielded a little batyl alcohol from methyl alcohol solution, and Zone 2 a little sterol, but most of this fraction consisted of the unsaturated selachyl alcohol, $C_{21}H_{40}O_3$. When all crystallisable material had been removed, as described above, with the aid of methyl alcohol, the residue was catalytically hydrogenated, giving a quantitative yield of batyl alcohol. The high iodine number of the liquid recovered from the filtrate suggested the presence of squalene, the hydrochloride of which was isolated by treating the solution in dry acetone with hydrogen chloride. The squalene hydrochloride formed characteristic crystals of melting-point 112° to 114° C.

Chromatography of the unsaponifiable fraction of fats thus assists materially in isolation of the components present.

3. Application to Pharmacy. Drugs and Galenicals

Just as extracts of natural tannins can be studied with the aid of chromatography, so can extracts of physiologically active drugs, galenicals, wines and similar substances. The chromatogram or ultra-chromatogram frequently gives information as to the method of preparation and the quality of such materials, and not infrequently assists in the detection

of sophistication. Sometimes the activity of a drug can be measured quantitatively by this means.

Valentin (1) first demonstrated the usefulness of Tswett's method in pharmacy when he chromatographed balsam of Peru and tincture of digitalis on a column of alumina.

BALSAM OF PERU was chromatographed both from a 0·5 per cent alcoholic solution and from a light petroleum solution (the

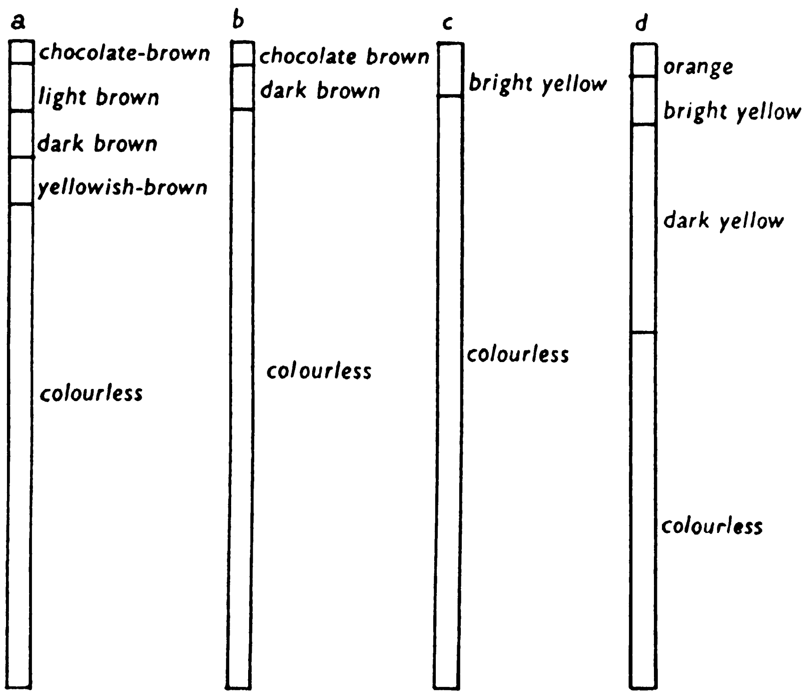


FIG. 40.—Chromatogram of balsam of Peru in ordinary light: (*a*) natural, and (*b*) artificial, balsam of Peru in alcohol; (*c*) natural, and (*d*) artificial, balsam of Peru in light petroleum

latter solvent left a residue of insoluble material). The difference in the appearance of the columns with natural and artificial balsams is illustrated in Figs. 40–1.

DIGITALIS LEAVES were extracted both with absolute alcohol and with dilute alcohol; the resulting chromatograms are illustrated in Fig. 42. Similar chromatograms from *Tinctura digitalis* were described and illustrated by Franck.

TINCTURE OF CANTHARIDES when chromatographed according to the conditions described by Valentin and Franck gave the

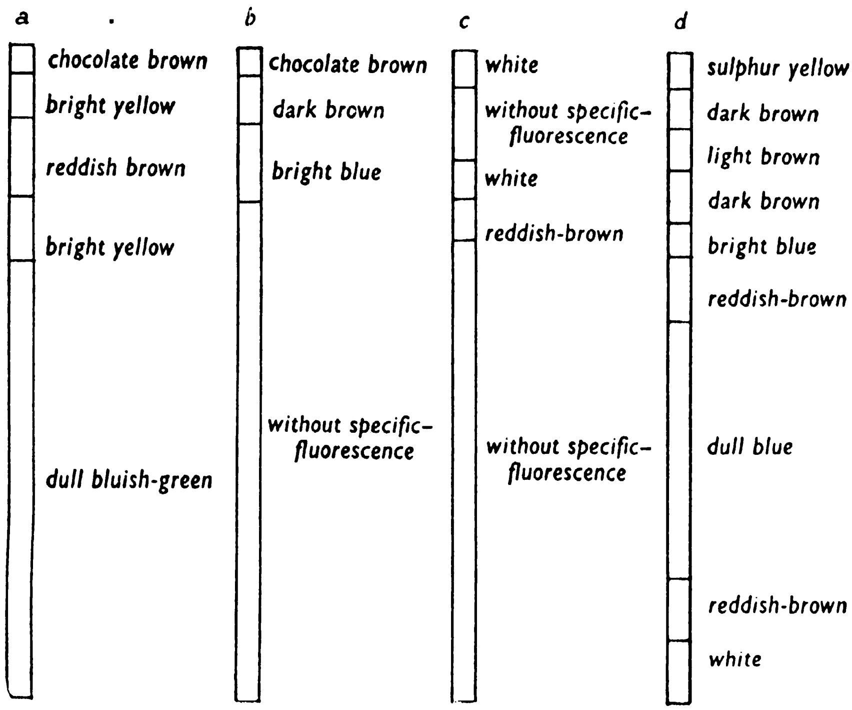


FIG. 41.—Chromatogram of balsam of Peru in ultra-violet light: (*a–d*) as in Fig. 40

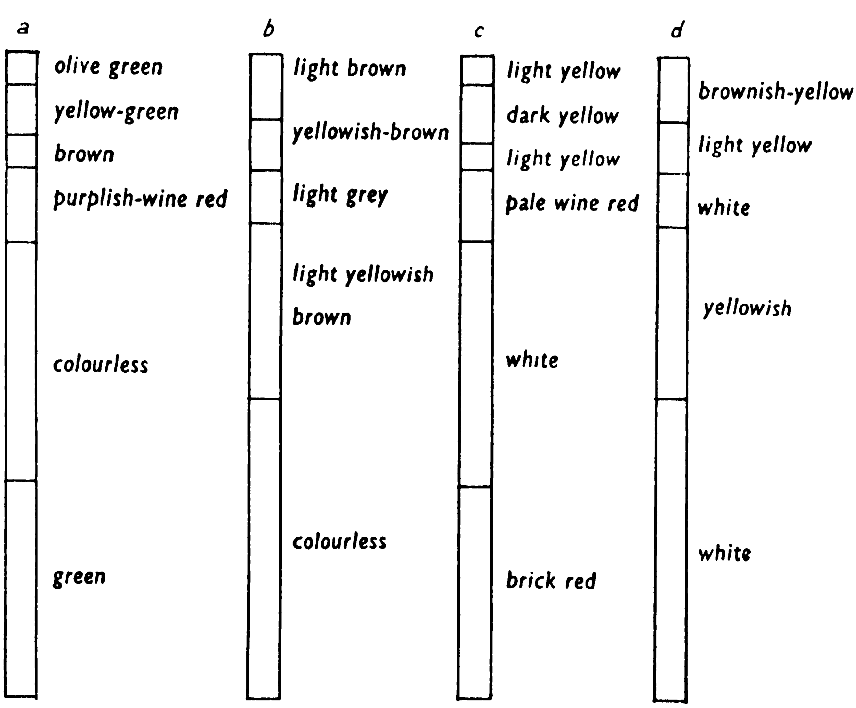


FIG. 42.—Chromatogram of tincture of digitalis: (*a*) prepared with absolute, (*b*) with dilute alcohol, in ordinary light; (*c*) prepared with absolute and (*d*) with dilute alcohol, in ultra-violet light

chrommatograms illustrated in Fig. 43 *a–b*, whereas extracts made from unsatisfactory or adulterated material gave chromatograms that were quite different in appearance (Fig. 43 *c–d*). The authors recommend the following method for the total estimation of the tincture.

The tube shown in Fig. 61, page 320, is filled with a thick suspension of alumina (Merck) in acetone, the excess of solvent

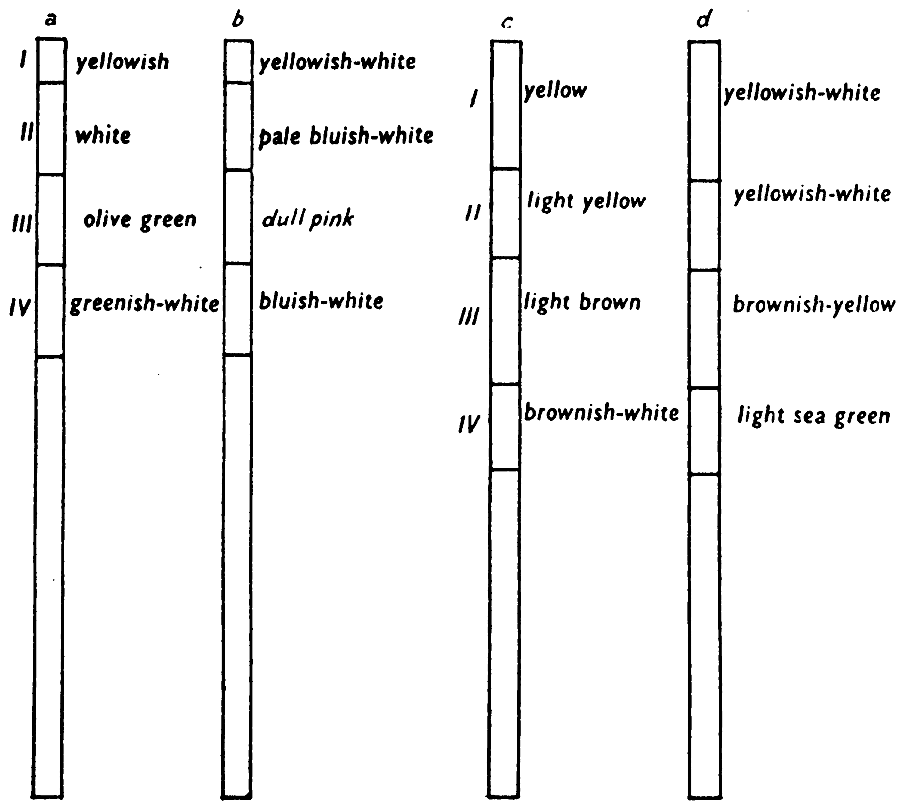


FIG. 43.—Tincture of cantharides, (*a–b*) according to the directions of the Pharmacopœia, (*c–d*) prepared by a non-official method; (*a*) and (*c*) in ordinary light, (*b*) and (*d*) in ultra-violet light

is drawn off, and the small amount of alumina that washes through is removed. Twenty grams of the tincture are then drawn through the column at a rate not exceeding 2 drops per second. The column is washed first with 10 ml. of a 1 : 1 mixture of acetone and chloroform and then with 20 ml. of chloroform. Cantharidin washes through into the filtrate, and the column should show the correct zoning, represented

diagrammatically in Fig. 43. The filtrate is transferred to a weighed flask with the aid of 5 ml. of chloroform and most of the solvent is distilled off. The remainder is removed by gentle heat and finally by a current of air. The residue is dissolved in a mixture of 9·5 ml. of light petroleum and 0·5 ml. of alcohol and the solution is allowed to stand for 12 hours. The crystals that deposit are filtered off and washed with four 5-ml. portions of the same mixture until the washings are colourless. They are then dissolved in 5 ml. of chloroform and the solution is returned to the original flask, in which it is evaporated to dryness. The residue is dried in a desiccator for 12 hours and

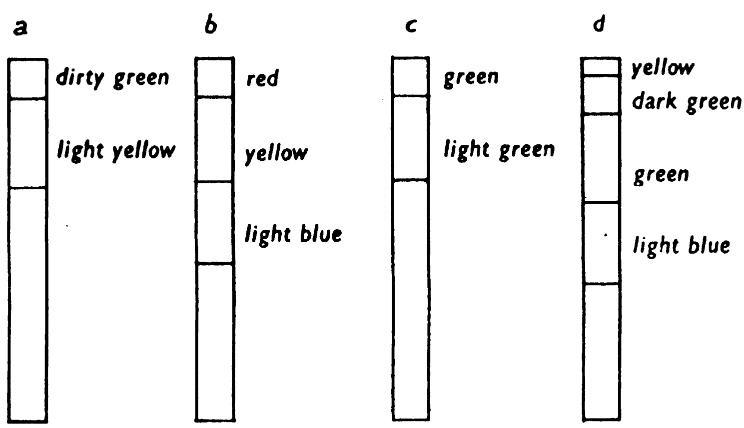


FIG. 44.—*Oleum Hyoscyami* from fresh leaves, (*a*) in daylight; (*b*) in ultra-violet light. *Oleum Hyoscyami* prepared by diluting "Ol. Hyoscyami decemplex," (*c*) in daylight; (*d*) in ultra-violet light

weighed. The white crystals of cantharidin, melting-point 211° C., should weigh at least 0·014 g., that is 0·07 per cent of the weight of the tincture taken for analysis.

OLEUM HYOSCYAMI: This, diluted with an equal volume of light petroleum, was chromatographed on alumina (Merck) and the column was washed with the same solvent. With the aid of the resulting chromatogram (Fig. 44) Franck was able to distinguish an extract of fresh henbane-leaves (made according to the directions of the Swiss Pharmacopœia) from a solution made by a tenfold dilution of a commercial concentrate.

SAFFRON: Adulteration with *flores calendulæ* can be detected chromatographically when, for instance, the extract obtained from 0·25 g. by means of 5 g. of 70 per cent. alcohol

is poured on to a column of alumina, which is then washed with the same solvent. The low-grade drug yields many more bands than the material of high quality (Fig. 45). The

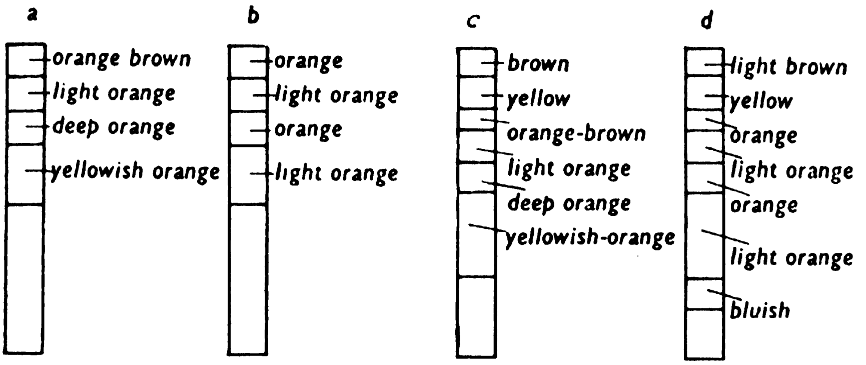


FIG. 45.—Crocus extract, (*a*) in daylight; (*b*) in ultra-violet light. Crocus adulterated with *Flores Calendulæ*, (*c*) in daylight; (*d*) in ultra-violet light

publication of Franck contains other examples of the same kind: *Vinum Condurango*, *Tinctura Absinthii*, *Tinctura Strophanthi* and *Tinctura Digitalis*.

Anthraquinone Drugs

The following method was adopted by Ernst and Weiner: An extract was prepared from 4 g. of the powdered drug by digestion with 40 ml. of cold 96 per cent alcohol for 24 hours. A column of magnesium oxide 25 cm. high and 1·3 cm. in diameter was prepared, and 1 ml. of water was added dropwise to it from a pipette, followed by 10 ml. of the extract, also from a pipette. The column was then developed by the dropwise addition of 10 ml. of alcohol and then 10 ml. of water. Magnesia, because of its alkalinity, has the advantage of causing the emodin-containing layer to be coloured red, at any rate in the topmost portion of the column (Table 23). The whole of the lower part of chromatograms of frangula, araroba, senna, etc., on the other hand, are coloured pale yellow, and have a greenish-yellow fluorescence in ultra-violet light; this zone contains anthranols. The fluorescent red zone of, for example, *fructus sennæ*, which overlaps the latter zone, contains chlorophyll.

Properties of the Magnesium Oxide Adsorbates

1. HYDROXY - METHYL - ANTHRAQUINONES : (*a*) Reddish-brown in daylight, with a reddish-brown fluorescence in ultra-violet light. (*b*) A red colour is formed when a little of the adsorbate is treated with a drop of potassium hydroxide solution. (*c*) A yellow solution is obtained on eluting with ether. When this is shaken with an equal volume of 5 per cent ammonia, the lower layer is coloured cherry red. (*d*) The residue obtained on evaporating the ether eluate gives yellow needles on micro-sublimation ; with potassium hydroxide solution, these crystals give a red solution. (*e*) Test for chrysophanic acid (1 : 8-dihydroxy-3-methyl-anthraquinone) : the adsorbate is heated with 15 per cent sodium hydroxide solution and filtered, and the filtrate is extracted with 3 volumes of light petroleum. Two millilitres of the yellow extract are then mixed with 2 ml. of ammonia ; on separating, the lower layer is coloured violet. (*f*) Test for frangula-emodin (1 : 6 : 8-trihydroxy-3-methyl-anthraquinone) : the adsorbate is heated with a little potassium hydroxide solution and filtered. The filtrate is acidified with dilute sulphuric acid and shaken with 3 volumes of benzene, into which the emodin passes, forming a yellow solution. On shaking 2 ml. of the solution with 2 ml. of ammonia, the latter is coloured purplish-red.

2. ANTHRANOLS : (*a*) Pale yellow in daylight, with a yellowish-green fluorescence in ultra-violet light. Vitreous appearance. (*b*) Aqueous eluate fluoresces yellowish-green. (*c*) The residue obtained by evaporation of a light petroleum extract of the adsorbate turns black when treated with a drop of Mecke's reagent (1·5 g. of selenious acid in 100 g. of conc. sulphuric acid). (*d*) Micro-sublimation of the above residue yields colourless needles which give a positive anthranol reaction.

The appearance of the chromatogram and ultra-chromatogram prepared from *Cortex frangulæ* enabled the fresh drug to be differentiated from the stored material, whereas alcoholic extracts of the same bark prepared by different methods gave much the same chromatograms. A column (30 × 2·5 cm.) of magnesium oxide was used, 40 ml. of the extract being poured

TABLE

CHROMATOGRAMS OBTAINED

(Ernst and

The figures on the left indicate the thickness of the zones in millimetres, by Wilhelm Ostwald, translated by J. Scott Taylor[1] (Winsor & Newton,

	Aloe	*Araroba depur.*	*Cascara sagrada*	*Cassia fistula*	*Frangulae Cort.*
In daylight	*150 yellow ne 3* *12* leaf-green ne 24 3 yellow ia 1 40 yellow ia 2 45 white MgO	40 red ni 7 10 orange ni 5 10 yellow ia 3 50 orange ia 4 70 orange ea 5 70 white MgO	10 red ie 8 10 yellow ia 5 Rest white MgO	5 red ie 8 3 orange ie 6 Rest white MgO	30 red ne 8 20 leaf-green ie 24 80 grey b 30 yellow ea 2 90 white MgO
In ultra-violet light (filter)	160 orange na 4 2 yellow na 1 3 ultramarine ea 15* 40 ultramarine ia 15 45 blue-green MgO	40 violet ni 12 10 ultramarine ni 13 10 orange ia 5 50 yellow na 3 70 leaf-green ea 24 70 blue-green MgO	10 orange ne 6 10 yellow na 1 5 leaf-green na 24 155 ultramarine ea 15† 70 blue-green MgO	8 orange ne 6 2 red ne 7 60 ultramarine ea 15* 80 red ea 33 100 blue-green MgO	30 orange ne 6 20 yellow na 1 20 ultramarine ea 15 90 yellow ea 1 90 blue-green MgO

* Strongly luminous. † Different shades. ‡ Including a few varied tion with *Rheum austriacum*

[1] See footnote

23

WITH ANTHRAQUINONE DRUGS

Weiner)

the data on the right refer to the nomenclature used in "Colour Science," Ltd., 1933).

	Rhamn. cath. Fr.	*Rhei Austr.* Rad.	*Rhei Chin.* Rad.	*Sennæ Folium*	*Sennæ Fructus*
In daylight	5 leaf-green ie 23 35 yellow na 3 120 grey b 30 yellow ea 2 60 white MgO	55 red ie 8† 70 orange ea 4§ 55 white 70 white MgO	80 red ie 8† 35 orange ea 4§ 65 white ‡ 70 white MgO	10 orange ne 4 10 yellow ne 3 1 red ie 8 4 red ea 8 130 leaf-green ia 24 20 yellow ia 1 75 white MgO	5 orange ne 5 3 yellow ia 3 2 red ie 8 10 red ea 8 55 grey b 100 yellow ea 2 75 white MgO
In ultra-violet light (filter)	40 yellow na 1 40 blue ea 16 10 violet ie 12 80 red na 8 10 leaf-green ea 24 70 blue-green MgO	55 red ne 9 50 ultramarine ie 13 17 leaf-green ea 22 3 ultramarine ea 15 55 ultramarine ea 13 70 green-blue MgO	80 red ne 9 25 ultramarine ie 13‖ 65 ultramarine ea 13 80 green-blue MgO	10 orange ne 4 15 orange ia 4 3 red ne 8 12 violet ea 12 80 red ne 8 40 orange ia 6 15 leaf-green ia 24 75 green-blue MgO	5 orange na 5 3 orange ia 4 2 red ia 8 50 ultramarine ea 14 100 red ia 8 15 ultramarine ea 14 75 green-blue MgO

streaks. § Consisting of light and dark streaks. ‖ Obvious adultera-
or inferior material (Tientsin)

on p. 300.

on to it. After being developed with 50 ml. of alcohol and then with 50 ml. of water, the column had the appearance given in Table 24.

TABLE 24

ALCOHOLIC EXTRACT OF *CORTEX FRANGULÆ* ON MAGNESIUM OXIDE (Ernst and Weiner)

The figures on the left indicate the thickness of the zones in millimetres, the data on the right refer to the nomenclature used in " Colour Science," by Wilhelm Ostwald, translated by J. Scott Taylor.[1]

Drug after Storage		Fresh Drug	
Daylight	Ultra-violet Light	Daylight	Ultra-violet Light
15 red ni 7	15 orange ni 6	20 red ni 7	10 grey i
6 orange ni 6	8 orange ni 4	5 orange ni 4	15 yellow ni 2
4 orange ni 4	5 yellow na 1	15 violet ni 12	5 leaf-green ni 23
5 yellow ea 2	1 orange ni 4	25 leaf-green ie 23	8 orange ni 4
110 yellow ea 2	2 yellow ni 3	5 sea-green ia 21	2 yellow ie 3
110 white	4 ultramarine ea 15	80 leaf-green ea 24	10 violet na 10
	35 violet ea 10	110 yellow ia 2	50 red ia 7
	70 MgO		50 leaf-green ea 22
	110 yellow ea 1		110 yellow ia 1
50 MgO	50 MgO	40 MgO	40 MgO

[1] *Note :* The Ostwald system of colour nomenclature is based on the principle that any shade or tint of a colour can be expressed in terms of " greyness " and " full colour." Shades of grey are made by mixing varying proportions of pure white and pure black, and these proportions are indicated by means of the letters a (89 : 11) c, e, g, i, l, n and p (3·5 : 96·5). Mixtures of grey and full colour are designated by a combination of these letters together with a number. The number indicates the colour, the chromatic circle being divided for this purpose into 24 sectors as follows : (in the original German text, the circle was divided into 100 sectors, but the English translation has been followed in the above table), yellow 1–3, orange 4–6, red 7–9, purple 10–12, ultramarine blue 13–15, turquoise blue 16–18, sea-green 19–21 and leaf-green 22–24. The combination of letters indicates the proportion of grey and full colour. Thus the letters ec, ca, ea, . . . denote a high proportion of white and therefore a pale shade ; the letters pn, pl, nl . . . a high proportion of black, and therefore a dark shade ; and the letters pa, na, la . . ., pc, pi . . ., nc . . . a high proportion of full colour and therefore a bright shade.—TRANSLATORS.

Estimation of the Alkaloid Content of Galenicals

Merz and Franck adsorbed a whole series of galenicals, prepared according to the method of the German Pharmacopœia, on columns of alumina and, after developing them with 70 per cent alcohol, obtained characteristic chromatograms and ultrachromatograms. (See Figs. 46–9.) The quantitative estimation of the alkaloid content, which is greatly facilitated by the chromatographic method, can be carried out in half an hour. Under the conditions employed, the bases pass through into the filtrate and can be titrated directly, e.g., in the analysis of

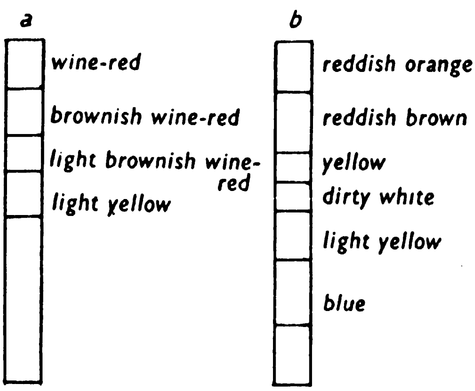


FIG. 46.—*Tinctura Chinæ*, (*a*) in daylight; (*b*) in ultra-violet light

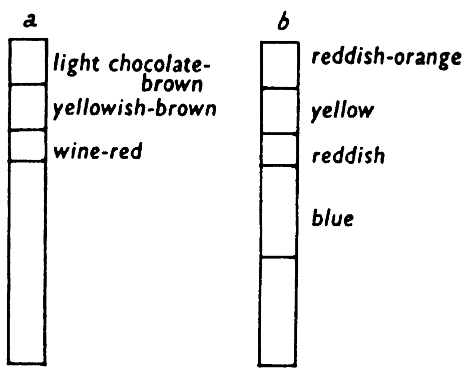


FIG. 47.—*Extractum Chinæ* spir., (*a*) in daylight; (*b*) in ultra-violet light

Tinctura Belladonnæ. The apparatus illustrated in Fig. 13 was used, the tube being filled with alumina (pure, anhydrous, Merck). Brockmann alumina was not satisfactory.

EXTRACTUM CHINÆ SPIR.: A solution of 0·5 to 1 g. of the extract in 10 ml. of 70 per cent alcohol was chromatographed and the column was washed with 70 per cent alcohol. The first 50 g. of the filtrate were collected and evaporated until turbid, and the residue was then taken up in 10 ml. of 0·1 *N*-hydrochloric acid and back-titrated with 0·1 *N*-sodium hydroxide solution, with methyl red as indicator.

EXTRACTUM CHINÆ FLUIDUM: Ten millilitres of the preparation were chromatographed and the column was developed with 40 ml. of 70 per cent alcohol. The first 50 ml. of the filtrate were treated as above.

TINCTURA STRYCHNI : Ten grams of the tincture were put on to the column, which was developed with 70 per cent alcohol until 40 ml. of filtrate had been collected. This was treated in the same way as the filtrate from *Extractum Chinæ spir.* The chromatogram showed a dirty-brown zone and a bright yellow zone ; in ultra-violet light it showed the following succession of zones : light brown, yellow and pale blue.

TINCTURA IPECACUANHÆ : Ten millilitres of tincture were chromatographed, and the column was developed with 70 per cent alcohol until 50 ml. of filtrate had collected ; this was treated as above (Fig. 48).

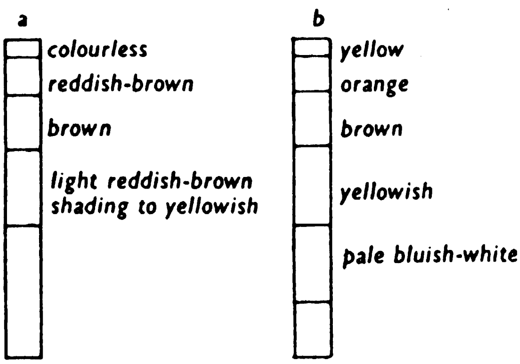


FIG. 48.—*Tinctura Ipecacuanhæ,* (*a*) in daylight ; (*b*) in ultra-violet light

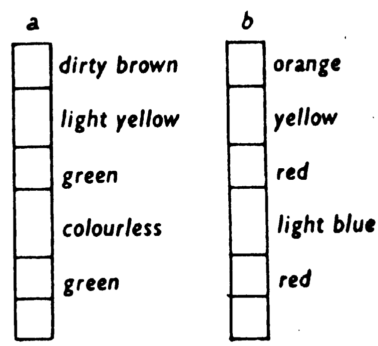


FIG. 49.—*Tinctura Belladonnæ,* (*a*) in daylight ; (*b*) in ultra-violet light

TINCTURA BELLADONNÆ : Ten millilitres were chromatographed and the column was developed with 40 ml. of absolute alcohol (Fig. 49). The filtrate (50 ml.) was evaporated on the water-bath and the residue was treated with 50 ml. of ether and then shaken with 3·5 g. of ammonia. The ether layer was run off and evaporated, leaving behind the free alkaloid, which was titrated in the usual way.

INFUSION OF COFFEE : Valentin (2) investigated the applicability of the adsorption method to the differentiation of various kinds of coffee, but obtained negative results, for different starting-materials gave chromatograms having very similar appearances (Fig. 50). Ten grams of the roasted, finely ground coffee were treated with 100 ml. of boiling water, and the mixture was boiled for 5 minutes, cooled and filtered. Ten millilitres of the filtrate were run through a column of

alumina (Merck), which was washed with 40 ml. of hot water. For estimating the amount of caffeine present, the combined filtrate and wash-liquors were evaporated in a porcelain dish and the residue was extracted four times with 10-ml. portions of chloroform. The combined extracts were evaporated in a tared flask and the residue was dried at 100° C. and weighed after cooling in a desiccator. A rough idea of the amount of

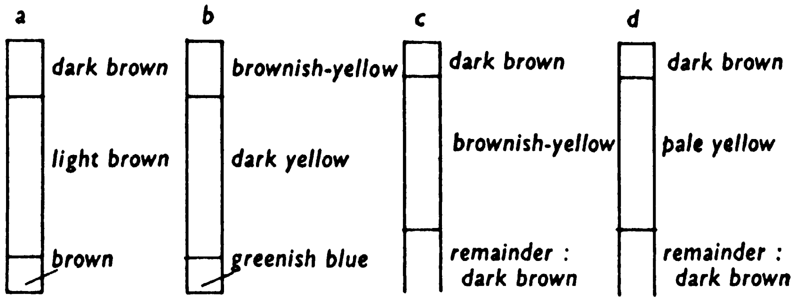


FIG. 50.—Chromatograms of coffee infusion (Valentin) : (*a*) genuine coffee in daylight ; (*b*) the same in ultra-violet light ; (*c*) malt "coffee" in daylight ; (*d*) the same in ultra-violet light

caffeine present could be obtained from the appearance of the column, for the normally bright violet fluorescence of the lowest zone is exceedingly weak when derived from caffeine-free preparations.

By treating the chromatogram with 1 per cent ferric chloride solution or with ferric chloride followed by 1 per cent sodium hydroxide solution, the appearance of the column is essentially changed. Details of this are given in the original paper.

CHAPTER 7

INORGANIC CHROMATOGRAPHY

(See Figs. 70–74, pp. 323–324.)

The fractionation of inorganic substances in the Tswett column has been investigated in comparatively recent times, and even now only a very small portion of the field has been explored. The method, however, has great possibilities.

The chromatographic method was recommended by Lange and Nagel for the separation of the rare earths, but, so far as we are aware, this suggestion has not been put into practice. Recently Schwab and Jockers (1, 2) described successful experiments, in which the detection and separation of ter- and bi-valent metal ions was attempted, whilst Schwab and Dattler (see also Schwab) studied the behaviour of a number of anions in the adsorption column. The results obtained by these workers are summarised in the following pages.

TECHNIQUE

The aqueous solution is poured on to alumina and the column is washed, usually with water, whereupon zones are either formed or they are more clearly differentiated from one another. Unfortunately, however, the regions between these zones are not usually colourless, and " development " with an appropriate reagent has to be effected in order to make the zones sufficiently distinct. Thus, for example, the detection of heavy metal ions is facilitated by " development " with ammonium sulphide or hydrogen sulphide solution or even gaseous hydrogen sulphide. Similarly, a ferricyanide or chromate zone is made manifest by the use of silver nitrate solution. A neat way of making colourless zones containing silver chloride visible is exposure to light, with the consequent formation of a violet colour.

Apparatus

In early experiments, the micro-tube of Hesse (1, 2), shown in Fig. 26, page 66, was used; it has an internal diameter of 4 to 7 mm. It is convenient, however, to have the tube widened out towards the upper end. The adsorbent, first freed from air by being heated at 70° to 80° C., is poured into the tube in the form of a thick, aqueous suspension, and is packed as tightly as possible by alternately applying strong suction and then tapping the tube vigorously. With molar solutions, the length of the tube must be so selected that the zone first formed occupies not more than one-seventh of the column. The passage of the liquid through the column takes place without suction.

Adsorbents

Both alumina (Brockmann) and ordinary dehydrated aluminium hydroxide can be used, the latter being just as good as the former. The material should preferably be of a fine granular nature. The following have been shown to be not very satisfactory adsorbents: barium sulphate, tin dioxide, titanium dioxide, thorium dioxide, beryllium oxide, powdered glass, powdered quartz, magnesium oxide, zinc oxide, cellulose and bakelite. Many of the bleaching-earths are unsuitable on account of their colour, whilst silicic acid varies in behaviour according to its mode of preparation, the most suitable type being made from silica gel.

Washing and Development

A volume of wash-liquors equal to 10 times that of the solution to be analysed is desirable when working with molar solutions, but with $M/10$ solutions the amount can be reduced to 3 or 4 volumes. The compound used as a developer should possess a smaller affinity for the adsorbent than the ion to be detected. Ammonium sulphide solution should be fully saturated with hydrogen sulphide; otherwise ions such as copper and silver will form complexes with the excess ammonia. On the other hand, with a solution containing a large excess of

hydrogen sulphide, the column is frequently disturbed by the liberation of bubbles of gas.

SEPARATION OF CATIONS

The most important bi- and tervalent metal ions, together with the mono-valent ions Ag^+ and Tl^+, are adsorbed on a column of alumina from aqueous solution in the following order :

Top :	As^{+++}
	Sb^{+++}
	Bi^{+++}
	Cr^{+++}, Fe^{+++}, Hg^{++}
	UO_2^{++}
	Pb^{++}
	Cu^{++}
	Ag^+
	Zn^{++}
	Co^{++}, Ni^{++}, Cd^{++}, Fe^{++}
	Tl^+
Bottom :	Mn^{++}

It can be seen that tervalent ions possess the strongest adsorption affinity in the series. The chromic, ferric and mercuric ions on the one hand, and the cobalt, nickel, cadmium and ferrous ions on the other, cannot readily be separated from one another. Thus when two or more members of either of these groups are present in a mixture, they form mixed zones not easily differentiated. In general, the further apart they are in the adsorption series, the more sharply and easily can two metals be separated.

The following examples are cited to illustrate the behaviour of mixtures towards chromatographic separation.

1. A molar solution of ferric, cupric and cobalt nitrate was chromatographed on alumina and the column was washed with water.

Top :	brown : iron
	blue : copper
Bottom :	pink : cobalt

After development with potassium ferrocyanide solution, the column had the appearance :

Top :	Prussian blue : iron
	brown : copper
Bottom :	greenish : cobalt

2. A solution of the nitrates of lead, silver, zinc, cadmium and manganese was chromatographed on alumina and the column was washed with water and developed with ammonium sulphide solution, forming the following chromatogram :

Top :	black : lead
	grey : silver
	white : zinc
	yellow : cadmium
Bottom :	flesh-coloured : manganese

In Table 25, page 308, criteria are given for the characterisation of different pairs of metals that have similar adsorption affinities and are therefore not sharply separated on the column.

Notes on Table 25

No. 1 : The column must be acidified with hydrochloric acid to prevent precipitation of basic salts. An obvious disadvantage of such an acid column is that cations are much less firmly held than with an ordinary column. Development proceeds slowly, because of the small amount of hydrogen sulphide that is present in an aqueous solution. For this reason, only small amounts of solution should be taken for analysis. After developing, two quite distinct zones are seen, the upper one yellow, due to arsenic sulphide, the lower orange-red, due to antimony sulphide. The zones can be completely separated by prolonged washing with dilute hydrochloric acid.

No. 2 : A clean separation between the upper, orange-red zone of antimony sulphide and the lower, brownish-black zone of bismuth sulphide is obtained on developing. The bismuth passes down the acid column so quickly, however, and development with hydrogen sulphide solution takes place so slowly, that only the last traces of the bismuth are retained on the column.

No. 3 : In order to prevent the possible precipitation of basic bismuth nitrate on first adding the solution to the column, it is advisable to make the upper part of the column slightly acid. A greenish-grey ring is first formed, but disappears on washing the surface of the column with a little acid and water. The column must then be washed with ammonia before being developed with ammonium sulphide solution, as otherwise

TABLE 25

SEPARATION OF ADJACENT PAIRS OF CATIONS ON A COLUMN OF ALUMINA (Schwab and Jockers 2). *M* to *M*/10 solutions. For notes, see pp. 307–311.

No.	Cations	Compounds used	Column	Wash-liquid	Developer
1	As^{+++} and Sb^{+++}	$AsCl_3 + SbCl_3$, treated with tartaric acid, and diluted with water	Acidified with dilute HCl	Dilute HCl	H_2S-water
2	Sb^{+++} and Bi^{+++}	$SbCl_3 + Bi(NO_3)_3$, treated with tartaric acid, and diluted with water	Acidified with dilute HCl	Dilute HCl	H_2S-water
3	Bi^{+++} and Cr^{+++}	$Bi(NO_3)_3 + Cr(NO_3)_3$, diluted with dilute HNO_3	Topmost zone acidified with a few drops of dilute HNO_3	First a few drops of dilute HNO_3, then water	NH_3 and $(NH_4)_2S$
4	Bi^{+++} and Fe^{+++}	$Bi(NO_3)_3 + Fe(NO_3)_3$ diluted with dilute HNO_3	Topmost zone acidified with dilute HNO_3	First a few drops of dilute HNO_3, then water	NaOH and $(NH_4)_2S$
5	Bi^{+++} and Hg^{++}	$Bi(NO_3)_3 + Hg(NO_3)_2$ diluted with a little dilute HNO_3	Acidified slightly at the topmost end	First a few drops of dilute HNO_3, then water	NaOH and $(NH_4)_2S$ or H_2S
6	Cr^{+++} and UO_2^{++}	$Cr(NO_3)_3 + UO_2(NO_3)_2$	Not treated	H_2O	$(NH_4)_2S$
7	Fe^{+++} and UO_2^{++}	$Fe(NO_3)_3 + UO_2(NO_3)_2$	,, ,,	,,	$K_4[Fe(CN)_6]$
8	Hg^{++} and UO_2^{++}	$Hg(NO_3)_2 + UO_2(NO_3)_2$	,, ,,	,,	$(NH_4)_2S$
9	UO_2^{++} and Pb^{++}	$UO_2(NO_3)_2 + Pb(NO_3)_2$	,, ,,	,,	,,
10	Pb^{++} and Cu^{++}	$Pb(NO_3)_2 + Cu(NO_3)_2$	,, ,,	,,	,,
11	Cu^{++} and Ag^{+}	$Cu(NO_3)_2 + AgNO_3$	,, ,,	,,	NaOH
12	Ag^{+} and Zn^{++}	$AgNO_3 + Zn(NO_3)_2$	,, ,,	,,	,,
13	Zn^{++} and Cd^{++}	$Zn(NO_3)_2 + Cd(NO_3)_2$	,, ,,	,,	$(NH_4)_2S$

evolution of gas takes place, sufficient to disturb the uniform packing. On development with ammonium sulphide solution, an almost white zone is produced at the top, and below it the bismuth sulphide zone, which ends where the greenish-grey ring of chromium hydroxide begins. The white ring at the top is formed by the free acid present in the original solution.

No. 4 : The iron zone is first noticeable in the basic part of the column. On development with ammonium sulphide solution, a white zone is again formed at the top, with the dark brown zone of bismuth sulphide immediately below it. Further down the column is the zone of ferric iron, which is converted into greenish-black iron sulphide by means of ammonium sulphide. On washing the column with dilute sulphuric acid, the iron sulphide zone is dissolved, but the bismuth sulphide remains unaffected.

No. 5 : On development with sodium hydroxide solution, a yellow band of mercuric oxide is produced ; this is separated from the surface of the column by a broad white zone. On now adding ammonium sulphide or hydrogen sulphide solution, this white zone becomes deep brown, with the exception of a narrow band right at the top, where free acid has been adsorbed, and the yellow zone becomes black. Hydrogen sulphide drawn through the column as gas is better than a solution, because distortion of the zones formed in the preliminary fixation with sodium hydroxide is thereby avoided. When ammonium sulphide solution is used, a greyish-black portion detaches itself from the mercury zone and is washed out of the column.

No. 6 : A greenish-grey zone occupies the top of the column and usually merges into a yellow zone immediately below it. The separation is incomplete, but can be improved by more prolonged washing. Development has no effect on the greyish-green zone of chromium hydroxide, but the lower zone is changed to the brown colour of uranyl sulphide.

No. 7 : On washing, a brown ring is produced at the top and a distinct yellow zone below it. On developing, these form Prussian blue and brown uranyl ferrocyanide respectively. Since, however, the precipitated ferric hydroxide adsorbs a part of the uranyl ion, the separation is not good when an excess of ferric nitrate is present.

No. 8 : A yellow ring that first forms is dissolved from the

surface. On prolonged washing, the white zone thus remaining becomes coloured yellow to red, as a result of hydrolysis of the mercury salt. On developing, the upper zone becomes black, due to mercury sulphide, and the lower yellow band brown, due to uranyl sulphide. Since, however, the mercury sulphide produced by ammonium sulphide is colloidal, and so tends to run through the column, it is better to fix the column with potassium ferrocyanide solution and then draw hydrogen sulphide gas through.

No. 9 : The yellow uranium zone is not dissolved from the surface. On developing, the usually brown uranyl zone becomes almost black, like the lead sulphide zone below it. Thus the separation is not complete, but it can be improved by adequate washing.

No. 10 : The blue copper zone is not entirely removed from the surface of the column, though most of the copper passes into the lower part. On developing, a black zone of lead sulphide is formed at the top, contaminated, that is, with a trace of copper sulphide. The greenish-black copper sulphide zone is formed lower down.

No. 11 : The blue copper zone is not dissolved from the surface. A brown ring of silver oxide is formed below the copper zone on developing.

No. 12 : The developing solution should be saturated with hydrogen sulphide. It produces a greyish ring of silver sulphide at the upper end of the adsorbent. The latter is divided just below the lower limit of the silver zone, and the top part of the rest of the column is digested with hydrochloric acid. The filtrate, after being neutralised with sodium acetate, gives a precipitate of zinc sulphide.

No. 13 : On developing, a white zone is formed at the top and a sharp band of cadmium sulphide immediately below it. Zinc can be detected in the upper zone as described above. Thus the presence of zinc is indicated by the appearance of a white zone between those of its two neighbours silver and cadmium, when these occur in a mixture together, since unoccupied white zones are never formed in inorganic chromatography. It is fortunate indeed that in the qualitative separation of the metals, the nature of the anion makes no difference at all to the order in which the metals are adsorbed (given on

p. 306). Also, where the presence of sparingly soluble salts prevents the method from being used, these can be dissolved with the help of free acid. The presence of foreign salts does not as a rule appreciably change the appearance of the column.

Exceptions : Silver and thallium are adsorbed abnormally low down the column when this is washed with a strong solution of potassium or sodium nitrate. Thallium may then be found below zinc, and silver in the zinc or even in the cobalt zone. This observation enables a sharp separation to be made between thallium on the one hand and cadmium or manganese on the other. This effect of nitrates takes place only on washing, and not when the column is merely moistened with a nitrate solution.

INFLUENCE OF THE BASIC CHARACTER OF THE ALUMINA COLUMN ON THE EXPERIMENTAL RESULTS : The disturbances arising from this factor are many and varied, as shown by the following examples :

(*a*) In the presence of free acid, a colourless zone is formed above the metal zone, owing to the adsorption of hydrogen ions. Metal ions are not adsorbed by this portion of the column.

(*b*) The method is not applicable to the detection of the mercurous ion, which is converted into free mercury and mercuric ions (the grey mercury zone). This change does not occur on an acidified column, but the mercurous ions then pass very quickly through the column.

(*c*) A neutral solution containing a manganous and a silver salt gives metallic silver and hydrated manganese dioxide. Similar reactions occur between cupric and ferrous ions, and between mercuric and palladous ions, so that one of the pair is oxidised and the other reduced. The difficulty can usually be overcome by adding to the mixture some other metal that occupies a place between the two in the adsorption series.

(*d*) The ferric ion is readily precipitated on the column as the hydroxide, and this is a source of trouble, because ferric hydroxide itself adsorbs readily. Thus a much smaller copper zone is formed when excess of iron is present than in its absence.

Separation of Cations as Ammine Complexes

If the water is replaced by ammonia, both for moistening the column and for washing it, then the order of adsorption is altered in a characteristic fashion. Thus, for example, silver, which forms a particularly stable ammine complex, occupies a lower position on the column than those ions which were previously below it :

Top :	Co^{++}
	Zn^{++}
	Cd^{++} Cu^{++}
	Ni^{++}
Bottom :	Ag^{+}

The use of such ammoniacal solutions is of value in the separation of cobalt from nickel, cobalt from cadmium, and cadmium from nickel, especially with the interposition of zinc.

Separation of Cobalt and Nickel

The mixture of nitrates, together with adequate zinc nitrate, is dissolved in excess of conc. ammonia, and the solution is poured on to a column prepared with conc. ammonia. The chromatogram is also washed with conc. ammonia. A brown cobalt zone is formed at the top and is separated by a white zinc zone from the nickel zone below it. On developing the column with colourless ammonium sulphide solution, the top and bottom zones become black, but remain separated by the white zinc zone. An effective separation of the cobalt and nickel is thus obtained by cutting the column across the middle of the zinc zone.

Separation of Cations as Tartrato-Complexes

As in the preceding instance, the order of adsorption is modified :

Top :	Mn^{++}
	Cd^{++}
	Zn^{++}, Pb^{++}, Cu^{++}, Bi^{+++}, Fe^{+++}, Cr^{+++}
	Co^{++}
Bottom :	Ni^{++}

The method is especially applicable to the clean separation of

ferric and chromic ions, which is not easy to accomplish with simple aqueous solutions, on account of the tendency of these metals to hydrolyse and form adsorptive precipitates.

Separation of Ferric and Chromic Ions

The mixture of nitrates is dissolved in sodium hydroxide solution containing basic tartrate. A solution of the basic tartrate is also used for preparing and washing the column. The chromium zone only is perceptible, and on washing it passes quickly from the surface into the lower part of the column. After developing with ammonium sulphide solution, a greenish-black zone of iron sulphide makes its appearance at the top of the column, becoming feebler below where it adjoins the green chromium zone.

Quantitative Experiments

It is obvious that the method has possibilities in micro-analysis for the detection in a metal of traces of elements that occupy a position above it in the adsorption series. Thus, for example, 0·2 ml. of a 0·0001 *M* solution, that is 1 μg, of ferric iron can be detected in a molar solution of another metal, such as copper or cobalt, by developing with potassium ferrocyanide solution. So also 1 μg of copper can be detected in a molar solution of cobalt or cadmium. The method employed is as follows : A series of dilutions of the trace-metal is prepared by successive tenfold dilution of a 0·01 *M* solution with a weakly acidic molar solution of a metal, such as iron or copper. Of each dilution, 0·2 ml. is poured on to a column (3 to 4 mm. internal diameter) of alumina, which is then developed with potassium ferrocyanide solution. The reason for acidifying the solution is to ensure the presence of a colourless zone at the top of the column, so that the coloured ring is more easily seen. The final dilution at which the impurity can still be detected gives a sharply defined ring 0·1 mm. wide, blue with iron and brown with copper, whereas the column prepared with the next dilution is indistinguishable in appearance from that of a control experiment without any impurity present. It is also conceivable that the chromatographic method may be of use in the macroscopic quantitative analysis of metals, since the

phenomenon apparently consists in the complete displacement of one ion by another. Moreover, the number of theoretical places utilised in the adsorption appears to be constant per unit length of column; from this and from the compression effect during adsorption, it follows that the length of a zone should be a measure of the amount of adsorbed substance. Although variable results have been obtained, it is nevertheless possible, using an empirical standard, to make a rough estimate of the percentage composition of a mixture.

Separation of Anions

Schwab and Dattler found that it was only possible to separate anions in certain instances, and a systematic analysis of a complicated acid mixture is not feasible by the method. The main reason for this is the impossibility of rendering visible a number of the colourless anions. The following adsorption series was obtained with a column of alumina previously treated with dilute nitric acid:

Top:	OH^-
	PO_4^{---}
	F^-
	$[Fe(CN)_6]^{----}$, CrO_4^{--}
	SO_4^{--}
	$[Fe(CN)_6]^{---}$, $Cr_2O_7^{--}$
	Cl^-
	NO_3^-
	MnO_4^-
	ClO_4^-
Bottom:	S^{--}

Preparation of the Adsorbent

N-Nitric acid (2·5 ml.) is drawn through a column (8 × 0·5 cm.) of alumina as quickly as possible, to avoid saturation with carbon dioxide. The excess of acid is then removed by washing with 2·5 ml. of water. After adding a solution of the alkali salts to be tested, the column is again washed through with water.

Separation of Phosphate and Fluoride (Na_2HPO_4 and NH_4F)

Development with a solution of silver nitrate in nitric acid gives a band of yellow silver phosphate, Ag_3PO_4, at the top of

the column. Alternatively, the addition of a solution of ammonium molybdate in nitric acid gives a yellow zone of phospho-molybdate.

Separation of Chromate and Sulphate (K_2CrO_4 and Na_2SO_4)

The adsorption of the chromate ion always gives rise to a yellow chromate band with an orange-coloured dichromate band below it, the proportion of the two ions depending on the *p*H of that particular portion of the adsorbent. With a mixture of chromate and sulphate, the diffuse yellow chromate zone extends into the white sulphate zone, and the sharply defined dichromate zone is below this. If silver nitrate solution is used for developing the chromatogram, the top zone (Ag_2CrO_4) is reddish-brown in colour, the sulphate zone is a pale red, and the lowest zone ($Ag_2Cr_2O_7$) is deep red-brown.

Separation of Sulphate and Ferricyanide (Na_2SO_4 and $K_3[Fe(CN)_6]$)

The yellow ferricyanide zone travels down the column on washing and is developed with silver nitrate solution. A white zone of sulphate is formed at the top, and an orange-coloured zone, sharply outlined, of $Ag_3[Fe(CN)_6]$ at the bottom.

Separation of Chromate and Chloride (K_2CrO_4 and NaCl)

On washing, a yellow band forms at the top (chromate) and an orange-coloured zone below it (dichromate). The former, on developing with silver nitrate solution, becomes reddish-brown (Ag_2CrO_4) and the latter a deep red-brown ($Ag_2Cr_2O_7$). On exposing the column to light, a violet zone forms below the dichromate zone (decomposition of AgCl). Between the dichromate and the chloride zones is a narrow white band, arising from the inhibitory action of chromate on the photolysis of silver chloride.

Separation of Ferricyanide and Chloride ($K_3[Fe(CN)_6]$ and NaCl)

The yellow ferricyanide is retained at the top of the column and is converted into an orange-coloured zone by the action of silver nitrate. The chloride is adsorbed lower down the column

and its presence is established by the formation of a violet colour on exposure to light after development with silver nitrate solution.

Purification of Inorganic Substances

The adsorption technique can be used with especial advantage when it is desired to remove from a solution small quantities of an ion that possesses a greater adsorption affinity than the main component. It is then retained in the upper portion of the column. Schwab and Jockers (2) subjected most of their reagents to a chromatographic purification, thereby removing traces of iron from lead, copper or aluminium. Traces of acids were removed in a similar way. Alkali metals cannot be removed in a column of alumina.

Further investigations in this field are eminently desirable, in order to find a substitute for the older rather cumbersome methods of analysis.

FIG. 51.

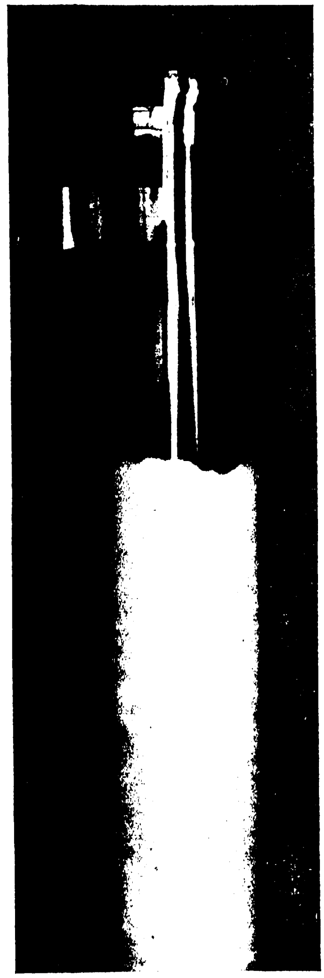


FIG. 52.

FIG. 53.

Simple chromatograms. Fig. 51 : zeaxanthin (above) and β-carotene (below) from petroleum on $Ca(OH)_2$. Example of displacement. Fig. 52 : a yellow zeaxanthin solution in a mixture (1 : 4) of benzene and petroleum on $CaCO_3$. Fig. 53 : the same column after the addition of a red capsanthin solution and development, whereby the zeaxanthin is displaced to a position lower down the column and its previous position is occupied by the capsanthin

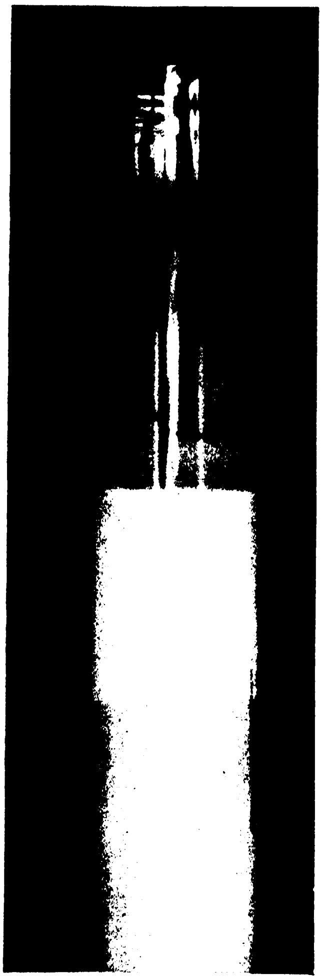


FIG. 54.

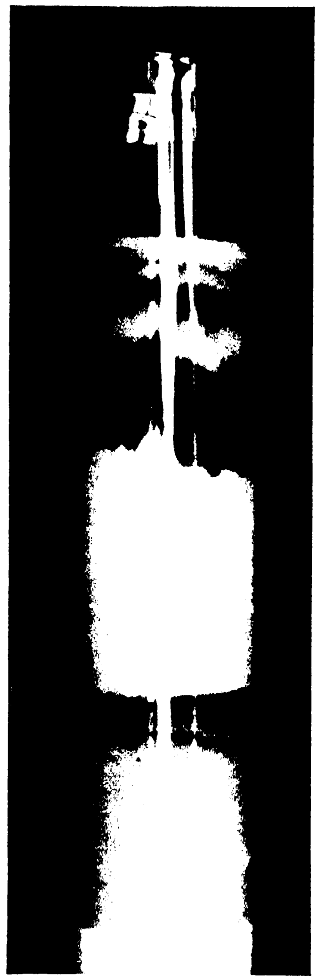


FIG. 55.

FIG. 56.

Formation of a chromatogram (crude extract of paprika, petroleum ; the tube filled with $CaCO_3$ above and $Ca(OH)_2$ below). Fig. 54 : immediately after pouring the solution on to the column. Fig. 55 : condition later, after inadequate development with petroleum. Fig. 56 : completely developed

FIG. 57.

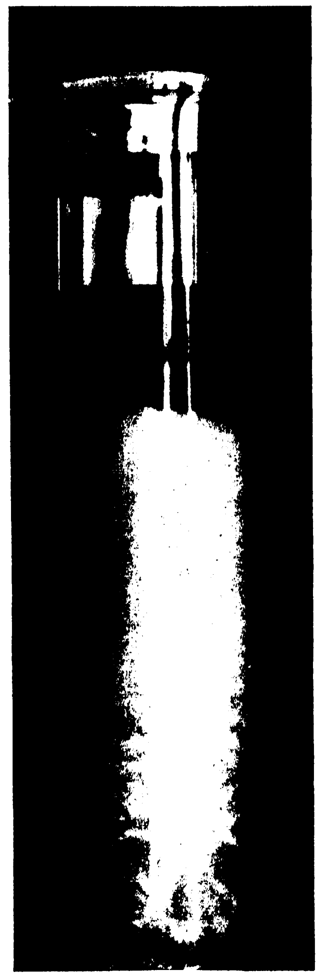


FIG. 58.

FIG. 59.

Unsuitable preparation of the column. Fig. 57: paprika extract as in Fig. 56, the adsorbent unevenly pressed down. Fig. 58: mixture of capsanthin and zeaxanthin (benzene, $Ca(OH)_2$) adsorbent badly selected, adsorption too strong; development made difficult. Fig. 59: the same in $CaCO_3$, adsorption too weak, pigment zone too diffuse, unfavourable ratio between the amounts of adsorbent and pigments. (Figs. 58–62 refer to the same pigment mixture)

FIG. 60.

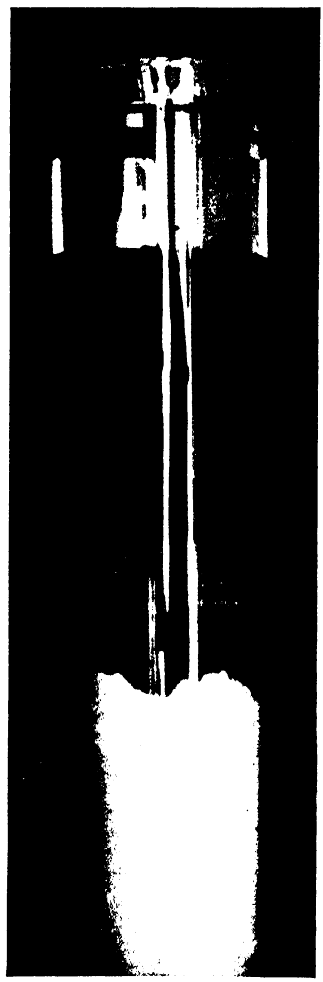


FIG. 61.

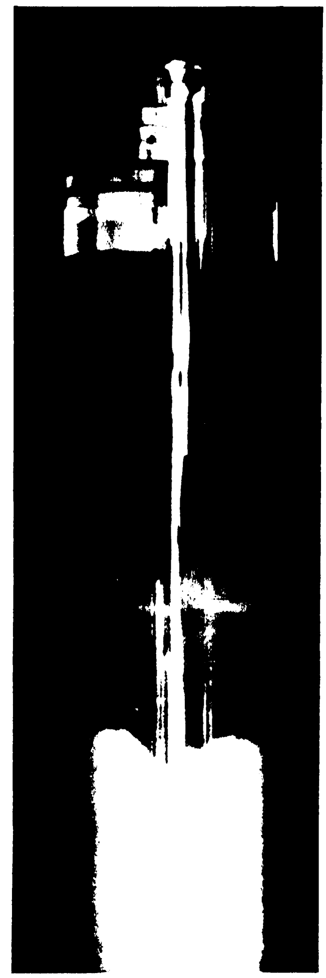


FIG. 62.

Influence of solvent and developing-liquid. Mixture of capsanthin and zeaxanthin on $CaCO_3$. Fig. 60: mixture (1 : 10) of benzene and petroleum, insufficient development. Fig. 61: mixture (1 : 4) of benzene and petroleum, correct development. Fig. 62: carbon disulphide, correct development. (Figs. 58–62 refer to the same pigment mixture)

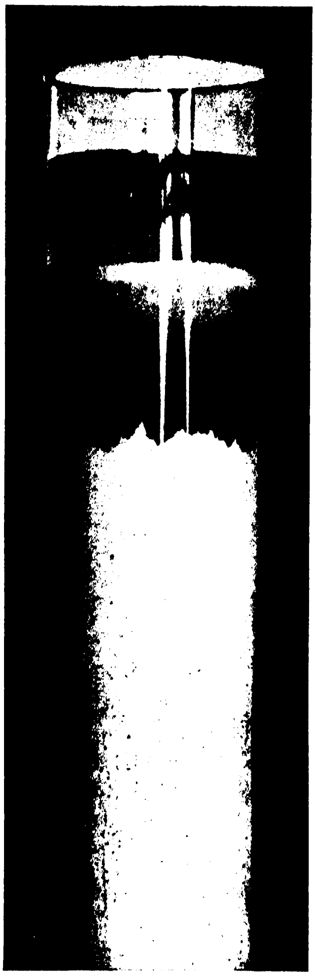


Fig. 63.

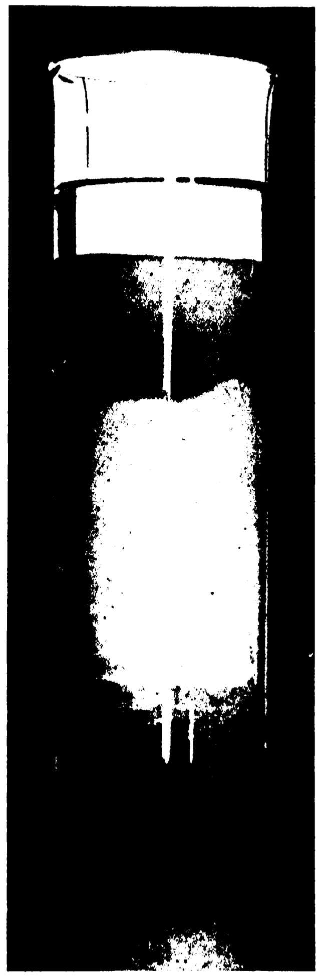


Fig. 64.

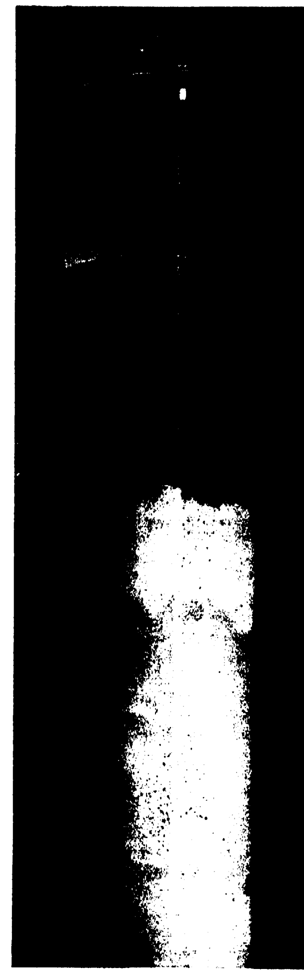


Fig. 65.

Influence of impurities. Fig. 63 : plant extract containing lycopene (petroleum, $Ca(OH)_2$). Fig. 64 : the same, mixed with a solution of lard before being poured on the column. Fig. 65 : crude extract of orange peel (CS_2, $CaCO_3$) : the natural constituents accompanying the lipochromes in this instance permit the immediate formation of finely differentiated bands

Fig. 66.

Fig. 67.

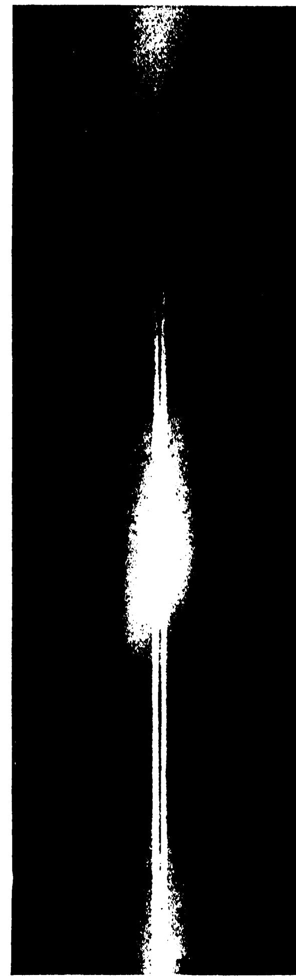


Fig. 68.

Detection of isomerisation. Fig. 66 : chromatogram of a freshly prepared solution of lycopene in benzene-petroleum mixture (3 : 1) on $Ca(OH)_2$; apart from a single unimportant constituent (band at the top), the chromatogram is homogeneous. Fig. 67: the same solution after being heated under reflux for 30 minutes, and then rechromatographed ; a new zone of neolycopene has made its appearance below the lycopene zone (see pp. 119 and 149, Zechmeister and Tuzson 16, 17). Fig. 68 : separation of *cis-* and *trans*-azobenzene from benzene solution in an alumina column (Zechmeister, Frehden and Fischer Jörgensen)

FIG. 69.—Brush method. Detection of vitamin A. The petroleum solution was chromatographed on $Ca(OH)_2$ and the extruded colourless column was painted with the Carr-Price reagent

FIG. 70.

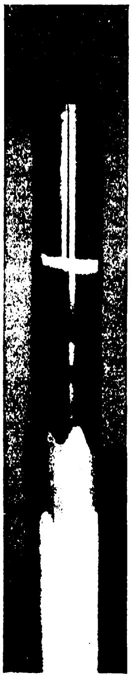


FIG. 71.

FIG. 72.

Separation of inorganic cations in the alumina column. Fig. 70 : Ferric, lead and silver nitrate, developed with NaOH ; top brown (Fe), middle greyish white (Pb), bottom black (Ag). Fig. 71 : Ferric, copper and silver nitrate, developed with NaOH, column not exposed to light ; top reddish-brown (Fe), middle blue (Cu), bottom bluish-grey (Ag). Fig. 72 : Ferric, cupric and cobalt nitrate, developed with potassium ferrocyanide ; top dark blue (Fe), middle brick-red (Cu), bottom greenish-grey (Co). (Figs. 70–2 are based on the publication of Schwab and Jockers 2)

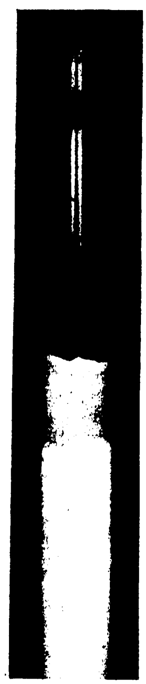


Fig. 73.

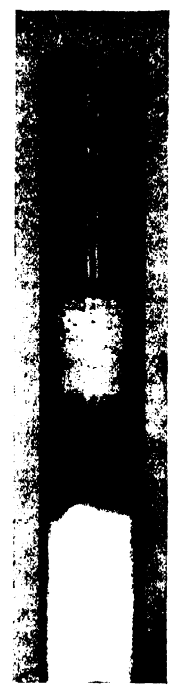


Fig. 74.

Separation of inorganic anions in the alumina column. Fig. 73 : Sodium chromate and sodium chloride, developed with $AgNO_3$; top dull red (silver chromate), bottom light grey (silver chloride). Fig. 74 : Potassium chromate and potassium ferricyanide, washed with water and not developed ; top yellow (chromate), bottom greenish (ferricyanide). (Figs. 73 and 74 are based on the publication of Schwab and Dattler)

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